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THE ADSORPTION OF SERUM ALBUMIN BY HUMAN ERYTHROCYTES

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The change of shape from biconcave disc to sphere which washed human erythrocytes undergo when they are run between a slide and coverslip of unwashed glass was investigated by R. F. Furchgott ('40) and by Furchgott and Ponder ('40) who ascribed this change in shape to the adsorption of a protein from the erythrocyte on to the glass surface; they considered that this loss of protein deprived the cell of its ability to maintain a discoid shape at the pH existing in the film of suspension — about pH 9.2. The protein held to be responsible for shape maintenance was called the "anti-sphering factor," and they brought evidence to show that it is the carbohydrate-poor fraction of the serum albumin known as crystalbumin (Hewitt, '38).

It would follow from such a hypothesis that washed human erythrocytes, brought into contact with a sufficient area of glass should, if the postulated loss of protein caused thereby is reversible, be able to remove crystalbumin from solution. This experiment was done (Furchgott and Ponder, '40) and nitrogen analyses by the micro-Kjeldahl method (no further specification is given) showed an apparent uptake of protein by the erythrocytes.

Furchgott and Ponder's experiments have been repeated, and further experiments have been carried out to test by a different method the hypothesis that human erythrocytes have a layer of protein at, or forming part of, their surface, which can be removed by contact with glass and replaced from solution.

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[1] Part of this work was carried out during an appointment with the Medical Research Council under Professor J. R. Squire.

METHODS

Throughout this work, blood was obtained by venepuncture from one subject (blood group B, Rh. positive), using a clean dry sterile syringe and mixed with sufficient heparin (B.D.H.) to prevent coagulation. The blood was centrifuged, the plasma removed and the cells washed 5 times in 10 times their volume of a $\frac{M}{9}$ solution of A.R. NaCl in glass-distilled water, the pH of which was usually 6.8. All experiments were done with freshly-drawn blood.

For the first set of experiments a large quantity of glass beads of about 3 mm diameter were taken, washed one hour in running tap water, stood overnight in chromic acid and washed again for 8 hours in running tap water. After this they were washed in 6 changes of glass-distilled water, and dried for 4 hours in an oven at 120°C. This cleaning procedure was used for all the experiments. Two glass tubes were now taken, each 4 cm in diameter and 1.4 metres long, and their bottom ends closed off except for an outlet tube and tap; they were clamped upright and filled with glass beads. It was calculated that the two tubes together contained about 12×10^4 beads, affording a total area of approximately 3.7 square metres. About 0.5 ml of lightly packed washed cells were suspended in a litre of saline, one half of which was poured slowly into each tube, until both were filled. After standing 10 minutes, the fluid was all run off into large centrifuge pots, the columns washed down with saline, and the recovered cells centrifuged. They were then pooled, and finally collected into about 7 ml of salt solution. Prior to this 50 ml of a solution of crystalbumin in $\frac{M}{6}$ NaCl had been placed in a centrifuge cup. The protein used was isolated from horse serum, and was 5 times recrystallized. Electrophoretic analysis showed it to be homogeneous, and very small quantities were able to reverse the shape change of human erythrocytes between glass slide and coverslip, just as effectively as human serum. Five millilitres of this solution was removed from the cup and transferred to a Kjeldahl digestion flask. Five millilitres of the treated cell suspension was added to the remaining 45 ml of protein solution and after mixing and standing for 5 minutes, the whole was centrifuged at 3,000 r.p.m. for 5 minutes. Then 5 ml of the cell-free supernatant was withdrawn and placed in a Kjeldahl digestion flask, and both samples digested, the procedure followed being that described by Peters and Van Slyke (Peters and Van Slyke, '32) except that heating was continued for one hour after the digest mixture cleared. Nitrogen was then estimated by the method described by Pregl (Pregl, '30). A haematocrit determination was made on a portion of the suspension of treated cells, and from this the volume of cells added to the supernatant was estimated.

For the second set of experiments about 0.5 ml of lightly packed cells were washed and suspended in 1 litre of a solution of $\frac{M}{10}$ NaCl, buffered by the addition of $\frac{M}{15}$ Na_2HPO_4 and NaH_2PO_4 at pH 7.2. The cell suspension was passed over the columns of glass beads and the recovered cells collected into a 10 ml graduated centrifuge tube and centrifuged so as to make an estimate of the volume of cells present. At this stage not more than 0.4 ml of cells was present.

Prior to this some of the horse serum crystalbumin already mentioned had been iodinated by the method of Li (Li, '45) using I^{131} and experiments with this material showed it to be a very effective "anti-sphering" agent. This radio-active iodinated protein was added to the suspension of glass-treated cells, the amount added being calculated from the data of Furchgott and Ponder to be equal to that theoretically adsorbed from the volume of cells present after passage over the glass beads. The final volume of the suspension of treated cells was brought to 10 ml, and the activity of the whole suspension measured in a Geiger-Muller counter. Then after separation of the supernatant and two washings, the activity of the cells and of the pooled supernatants was measured separately. A control experiment in which the cells were treated exactly as described but not passed over glass beads was also performed.

RESULTS

Five experiments of the first type were done and the results showed that, using the techniques described, it was not possible to establish with certainty the differences — from 0.019 to 0.040 mg N_2 — which Furchgott and Ponder reported in their 4 experiments.

It was found that analysis of the sample withdrawn from the protein solution after addition of glass-treated cells might show a difference of up to 2% above or below the theoretical value calculated from the determination on the sample taken before the addition of the cells. The detailed results of the 5 experiments are given in table 1.

TABLE 1

EXPT. NO.	N_2 CONTENT OF 1ST SAMPLE	VOL. OF CELLS ADDED	N_2 CONTENT OF 2ND SAMPLE		DIFFERENCE
			Calculated	Found	
	mg	*ml*	*mg*	*mg*	*mg*
1	1.638	0.40	1.490	1.526	+ 0.036
2	0.620	0.37	0.562	0.560	— 0.002
3	0.544	0.28	0.497	0.501	+ 0.004
4	0.734	0.39	0.669	0.654	— 0.015
5	1.536	0.35	1.398	1.420	+ 0.022

In view of the difficulties attending this particular experimental procedure — discussed in detail below — it was desirable to test the hypothesis in question by an independent method, and the second set of experiments already described was designed to do this. The results are given in table 2. All the figures given are corrected for background.

TABLE 2

	EXPT. 1	EXPT. 2	EXPT. 3	CONTROL
Counts/second of whole susp.	260	1272	638	263
Counts/second of supernatants	269	1125	615	274
Counts/second of twice-washed cells	6	20	9	2
Ratio $\frac{\text{activity in cells}}{\text{activity in supernat.}}$	1:45	1:56	1:61	1:137

DISCUSSION

The failure in technique which is evident in the results of the first set of experiments was not easily understandable at first, because analyses of pure ammonium sulphate showed the method as used to be capable of an accuracy of at least ± 0.5% on this material. This is a reasonable degree of accuracy, having regard to lack of experience of the operator, and would have sufficed in the present experiments. However, a study of the literature on protein-nitrogen determination by the Kjeldahl method provided good reasons for this state of affairs. There is ample evidence (Haurowitz, '50; Van Slyke, Hiller and Plazin, '48; Chibnall, Rees and Williams, '43; Kirk, '47; Miller and Houghton, '45) that many sources of error not appreciated before have been revealed by studies of the Kjeldahl method for protein-N_2 in the last 10 years or so, that it is a specialized technique of some complexity and even standard procedures may lead to serious errors. In the case of these experiments, there is the additional difficulty that no absolute standard is available by which to estimate the possible error of the experiment as a whole, because the ''theoretical'' value for the second sample is itself calculated from an estimate of unknown degree of accuracy on the first sample. It is apparent from the discussions given by the authors already mentioned that in my own experiments the digestion procedure may well have been at fault, even though a generally accepted method was being used. It seems possible, in the absence of any information as to technique, that the results of Furchgott and Ponder in 1940 may be open to the same criticism, and two points might be made in this connection.

1. Simple calculation shows that if a possible error of ± 2% is allowed for in the nitrogen determinations given in Furchgott and Ponder's paper, the range of difference possible between the first and second samples in each experiment, is shown in table 3 on next page.

In experiment 3 there could in fact have been a "surplus" of N_2 in the second sample, and of the other three experiments only No. 2 would maintain a possibly significant difference.

2. A surprising feature of the experiments under discussion is that the amounts of protein removed from the solution by glass treated red cells bear no relation to the quantity of cells. Cell volumes differing by 4% (0.25 ml to 0.24 ml) can apparently remove amounts of protein differing by 40% (2.44 mg to 1.45 mg). The authors attribute the very large variations in protein adsorbed by glass treated red cells to "the limitations of the micro-Kjeldahl analysis with such small amounts of nitrogen as were analysed for here." But a far higher order of accuracy is assumed in drawing support for their hypothesis from the experimental data.

TABLE 3

Expt. 1	— 0.005 mg	to	— 0.043 mg N_2
Expt. 2	— 0.019 mg	to	— 0.061 mg N_2
Expt. 3	+ 0.006 mg	to	— 0.045 mg N_2
Expt. 4	— 0.002 mg	to	— 0.050 mg N_2

It is evident from the results of the second set of experiments that they do not support the hypothesis under consideration. This hypothesis would have predicted that at least a substantial fraction, if not all, of the radio-activity would be found in the cell deposit, whereas in fact it contains a negligible proportion of the total activity. It might be argued against this that the cells were not properly freed of their "anti-sphering protein" and this argument deserves consideration. The volume of cells used in the second set of experiments was about 0.4 ml or less, and an area of about 3.7 square metres of glass was provided for the removal of protein from the cells. In the paper by Furchgott and Ponder the area of glass provided is given as 10 square metres for every 0.5 ml of cells, and the diameter of the beads was 3 mm. Their procedure was to pour a 1:800 suspension of cells evenly over the glass beads which were in a beaker. In the experiments already quoted, they used the washed cells from 1 ml of blood, and thus would have needed about 9 square metres of surface. It can easily be shown that this is equivalent to some 320,000 glass beads, which would occupy a volume of about 7 litres or so. Thus, pouring 400 ml of suspension into such a quantity of glass beads might very easily mean that much less than the full surface area was used — in fact, experience has shown that unless some sort of column was set up, it would be very difficult to ensure that such an enormous quantity of glass beads is in fact brought fully into contact with a relatively small quantity of cell suspension. There is in addition the difficulty caused by rapid drying of the liquid film when a small quantity is spread over such a large area. In my experiments the total volume occupied by the beads was about 3.5 litres, i.e., about 1.75 litres in each column, and only by filling the residual space completely with cell suspension was it possible to utilize the whole available surface of the beads. This of course has the advantage of utilizing the surface of the glass tubes themselves, contributing about 0.34 square metres of surface to the total. In all these calculations the area lost through contact between beads and between beads and the tubes is ignored — they are for comparative purposes only. Finally it may be said that the amount of glass beads used in these experiments was as much as could be handled with reasonable convenience.

Now the particular ratio of surface-area to cell volume chosen by Furchgott and Ponder appears to be quite arbitrary, and apart from the possibility that it may not actually have been attained, there are reasons for considering a smaller ratio to be adequate, assuming the hypothesis to be a valid one. Many observations have shown that all the cells in a 0.05 ml aliquot of a 1:500 cell suspension, enclosed between a plastic slide and a glass coverslip of 3 square centimetres area, are converted to spheres. This indicates that an area of 3 square metres of glass is capable of adsorbing the protein from at least 0.5 ml of cells, and in these experiments that ratio is easily realized. Moreover assuming a surface area of 163 μ^2

for the human erythrocyte (Ponder, '48) and that a red cell count of 5 million cells per cubic millimetre corresponds to an haematocrit of 42%, calculation of the surface area of 0.4 ml of cells gives a figure close to 0.8 square metres. Since this is only one quarter of the area of glass in these experiments, there seems to be little reason to suppose that the latter was inadequate.

However, apart from any such consideration, the results of the experiments with labelled protein are against the assumption that any protein at all was removed from solution by the cells. The small difference between the activity in the deposit in the control experiment and those in which a cell suspension was run over the glass beads is probably due to the fact that the latter deposits always contained microscopic fragments of glass which would of course adsorb some protein and be carried into the deposit.

There is an alternative possible explanation for the sphering of washed erythrocytes between a glass slide and coverslip, and the "anti-sphering" action of crystalbumin, which deserves attention. The sphering phenomenon is only seen if the glassware used is new and unwashed, thus providing alkali to raise the pH of the film. A fundamental point in the argument in the two papers by Furchgott and Furchgott and Ponder is that this rise in pH alone cannot account for the sphering phenomenon, because suspensions made alkaline to the same extent (pH 9.2) by the addition of NaOH to a bulk suspension do not show spherical cells — these are only produced at pH levels several units higher. Thus it is postulated that the contact with glass must involve some other factor, and the evidence already reviewed is amongst that brought to show that this is adsorption of a protein. If, however, a suspension is brought to pH 9.2, not with NaOH but with $NaHCO_3$, all the cells are sphered in a hanging drop preparation on a plastic coverslip (or an acid washed glass coverslip) at this pH, and even at pH 8.0. Now the substance which will be dissolved off new microscope glassware and cause increased alkalinity in a film of cell suspension is sodium carbonate, and this of course yields HCO_3^- and OH^- on hydrolysis. Thus, one has well-washed erythrocytes, which through washing in NaCl solutions have lost their bicarbonate, transferred from the bicarbonate-free solution in which they were washed at about pH 6.8, to an alkaline medium containing HCO_3^-. Under these circumstances, as has already been shown (Jacobs and Stewart, '41) there will be a catalysed exchange of Cl^- for OH, and the interior of the cell will become alkaline at a far greater rate than is possible in the absence of HCO_3^-. This will of course lead to an equally rapid loss of cell water. In these circumstances the interior of the erythrocyte can undergo a much greater change in pH than is possible in physiological conditions, and it can be argued that the subjection of the proteins of the cell membrane to this sudden and large change of pH is the cause of the spherical shape which the erythrocyte assumes (Brown, unpublished). This shape change is very rapid indeed, a fact which is consonant with the explanation suggested. The effect of adding serum albumin in such a situation would be to lower the pH, and also, by the binding of HCO_3^- ions (Haurowitz, '50) to reverse the shifts which had produced the spherical form, so exhibiting an "anti-sphering" action. At pH 9.2, serum albumin can bind base to the extent of 60×10^{-5} moles of NaOH per gram of protein (Cohn, '25). A solution of NaOH at this pH contains 1.7×10^{-5} moles NaOH per litre, and if one assumes a value of say 0.05 ml for the film between a glass slide and coverslip as used in Furchgott's experiments, this would give a figure of 8.5×10^{-10} moles NaOH present in the film. A milligram of serum albumin could bind 60×10^{-8} moles NaOH, and would also bind HCO_3^- specifically. It seems clear that one does not have to postulate any specific adsorption of the protein by the cells to account for its "anti-sphering" action. It is of interest to note that serum from the rabbit, horse, guinea pig and rat are all capable of exerting such an action towards human erythrocytes, as is also egg-albumin and a protein from horse-dandruff. This would be a rather surprising circumstance if a specific adsorption on to the cell was the cause, but quite understandable if a simple physico-chemical equilibrium was in question. The failure to find any relationship between cell volume and amount of protein removed from solution is likewise more easily understandable.

My thanks are due to Dr. Hugh Davson of University College London, and to Professor Gilding of Birmingham University for help and advice, and to Dr. P. Gell of the Department of Experimental Pathology of Birmingham University for his assistance in measuring the activity of the labelled protein.

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THE DIFFUSION OF GLUCOSE INTO THE HUMAN RED CELL[1]

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EIGHT FIGURES

INTRODUCTION

The penetration of glucose into human red blood cells has concerned many previous investigators. Masing ('14), Ege ('25), Fleischman ('28), Mond ('30), Bjering ('32) and Svedberg ('33) are a few of the earliest investigators who reported that glucose penetration into human erythrocytes did not follow the laws of simple passive diffusion. Most of these workers were, however, primarily interested in the equilibrium distribution of glucose between cells and environment. Only Bjering ('32) attempted a study of the rate of penetration. He found the diffusion rate markedly less when the concentration of glucose was raised. He also noted a similar lowering of the diffusion rate in an artificial system involving a collodion membrane.

Bang and Ørskov ('37) reported that, on increasing the concentration of glucose from 0.02 to 0.05 *M* in a suspension of human red cells and saline, the permeability constant was reduced 2.5 times.

Klinghoffer ('35, '40) found that, on addition of concentrated glucose solutions (above 2300 mg%) to human blood, only a limited amount of glucose penetrated the cells. With more dilute solutions (1500 mg%) the distribution between

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[1] This work was supported in part by the Whitehall Foundation.

[2] This study was submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

cells and environment was equal and complete in 30 minutes. On the other hand if dry glucose was added to the blood the approach to equilibrium was greatly accelerated, and the glucose was evenly distributed through the water of the cells and the environment, even though the glucose concentrations were as high or higher than in those experiments involving addition of concentrated glucose solutions.

Peters and Van Slyke ('46) concluded that the human red blood cell is freely permeable to glucose by simple diffusion. They believe much of the confusion in the literature has been caused by failure to recognize reducing substances other than glucose in the blood, thus causing considerable error in chemical analytical data. They also consider as sources of error improper methods of estimating cell volume and cell water, and failure to take precautions against glycolysis.

More recently, LeFevre ('48, '52) has claimed that the laws of simple diffusion cannot predict the volume changes which human red cells undergo in glucose-saline solutions. He postulates a simple carrier system involving the formation of a highly undissociated complex between the glucose molecule and some factor at the cell surface. His experimental curves are exactly described in terms of this simple carrier system. However, he reports a limiting concentration for the carrier at three-fourths of isotonicity (0.23 *M* glucose). In more concentrated solutions red cells will not swell to predicted volume as the system fails to operate.

Wilbrandt and Rosenberg ('52) suggest a more complicated process for the transport of glucose into the red blood cell. Their theory would involve two separate enzymatic systems: one for penetration from the exterior to the interior of the cell and the other from the interior to the exterior of the cell.

Widdas ('53, '54) also reports that simple diffusion laws cannot account for the volume changes which red cells undergo in glucose-saline solutions. He finds that the permeability constant for glucose decreases by a factor of fifteen times as its external concentration is raised from 0.03 to 0.15 *M*. He suggests a kinetic system of facilitated transport similar to LeFevre's to explain his results. However, he reports no limiting concentration at which this transport system fails to operate.

Although most of the above investigators agree that a process other than simple diffusion is involved in the penetration of glucose, there is no agreement upon the nature of this process.

In the light of this involved situation it was decided to study the rate of penetration of glucose into human red blood cells observing as nearly normal environmental conditions for the erythrocytes as possible. Presumably additional quantitative data obtained by a photoelectric densimeter technique together with direct chemical analysis data and hematocrit measurements should be useful in determining the nature of the process by which glucose penetrates human erythrocytes.

MATERIALS AND METHODS

In these investigations the penetration of glucose into the human erythrocyte was measured by two methods. Firstly, a photoelectric densimetric technique allowed a recording of the volume changes which the cells undergo in hypertonic glucose-saline solutions. Secondly, a chemical analytical method permitted a study of the disappearance of glucose from the plasma of concentrated whole blood suspensions to which known amounts of glucose had been added. Volume changes in these concentrated suspensions were followed by hematocrit techniques which have been described previously (Parpart and Ballentine, '43).

Densimetry

The densimeter used in these experiments was similar to that developed by Ørskov ('35) and Parpart ('35). A beam of light from a tungsten lamp running at constant voltage (6 volts, D. C.) passes through a rectangular heat absorbing glass-faced brass tank in which a cylindrical cell suspension chamber of 8 ml capacity has been fixed in the path of light. Water from a constant temperature bath is circulated in the tank to keep the contents of the suspension chamber always at 37° C.

After passing through this tank the light beam impinges on the cathode of a photoelectric cell (RCA 927).

The current generated in the photocell is passed through a Direct Current Amplifier (General Radio Type) whose output is recorded by the pen of an Esterline-Angus 5 milliampere automatic recorder of one second period. The line voltages to the tungsten lamp and to the amplifier are regulated by ''Sola'' constant voltage transformers. This direct recording method has proved very convenient for this type of work.

The suspension chamber is covered by a leucite cap through which is bored a small hole to permit the entrance of the glass rod from a motor-drive stirrer. The whole apparatus is set up in a constant temperature room. These precautions eliminate fluctuations due to evaporation from the suspension chamber and to the temperature sensitivity of the tubes of the D.C. amplifier.

Blood for densimeter measurements was drawn from normal males by lancet finger puncture. In some cases, the sample was defibrinated, and in others 4 mg of sodium citrate were used per cubic centimeter of blood as an anticoagulant. The blood was quickly strained through gauze into a glass-stoppered vessel which was rolled on an ice bath as the experiments proceeded. Because the erythrocytes have a tendency to go into ''rouleaux'' formations when human blood is permitted to stand at room temperature, this rolling procedure is absolutely essential if duplicate results are to be obtained. These rouleaux formations, or stacking of erythrocytes, make it difficult to obtain the same cell-plasma ratio in every 20 mm^3 blood sample drawn up for experimentation. Variations in this ratio results in variation in transmitted light levels.

It was also found that for reproducible results fresh blood samples should be drawn about every 4 hours. This seems to be in agreement with the observations by Bang and Ørskov

('37) and Meldahl and Ørskov ('40) that glucose diffuses more readily into human red cells when the blood is fresh.

In all experiments precisely 20 mm³ of fresh, unwashed whole blood were added to 7 cc of a glucose-saline solution in the suspension chamber.

The solutions were prepared in the following manner. Stock solutions of 0.3 *M* glucose in 0.17 *M* NaCl were prepared. The 0.17 *M* NaCl was buffered to a pH of 7.5 or 7.78 by a standard phosphate buffer (Na_2HPO_4–NaH_2PO_4) Parpart et al. ('47). The stock solution was diluted with either 0.17 NaCl-PO_4 or 0.34 *M* NaCl-PO_4 of the same pH.

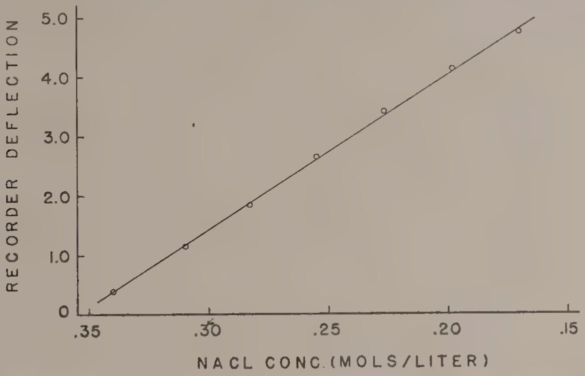

Fig. 1 Densimeter Calibration. A calibration of the relation between the volume of erythrocytes and the external osmotic pressure of the suspension fluid as recorded by the densimeter apparatus. Twenty cubic millimeters of whole blood is added to 7 cc of NaCl-PO_4 in the cell suspension chamber and the light intensity recorded by the Pen Recorder. Temperature 37° C., pH 7.78.

This procedure resulted in two sets of solutions: the first set varied in osmotic pressure according to the concentration of glucose present; as 0.1, 0.15, 0.20, 0.25 or 0.3 *M* glucose in 0.17 *M* NaCl-PO_4 while the other set consisted of solutions of approximately uniform osmotic pressure (two times isotonic) but which contained the same varying concentrations of glucose. In a few instances highly concentrated solutions of glucose were made up in 0.17 *M* NaCl-PO_4.

Figure 1 shows a calibration curve of the relation between the volume of red cells and the external osmotic pressure as recorded by the densimeter apparatus used in the experiments. The deflection of the pen recorder is plotted against the NaCl concentration of the suspension fluid whose pH is 7.78. Over this concentration range the volume of the red cell is linearly related to the reciprocal of the osmotic pressure (Parpart and Shull, '35a).

Chemical analytical

Normal human blood was drawn by venipuncture. Sodium citrate was used as the anticoagulant. The blood was strained through gauze, divided into 10 ml portions by volumetric pipette added to large 40 ml capacity glass-stoppered centrifuge tubes and prepared for experiment in the following manner:

Step 1 Cells and plasma were separated by centrifugation and two milliliters of plasma were removed from each tube for determination of the normal plasma glucose.

Step 2 The cells and plasma remaining in the 40 ml tubes were remixed by gentle rolling.

Step 3 One milliliter of the resulting concentrated whole blood was drawn up in a volumetric pipette and added to a small (15 ml capacity) test tube which was tightly stoppered.

Approximately 7 ml of concentrated whole blood remained in the 40 ml tubes.

Step 4 Cells and plasma in these 40 ml tubes were separated by centrifugation, and the plasma almost completely drawn off and placed in glass-stoppered vessels.

Step 5 Various amounts of glucose were now dissolved in the plasma samples obtained in Step 4.

Step 6 At this point the concentrated whole blood obtained in Step 3, the packed cells obtained in Step 4, and the plasma-glucose solutions resulting from Step 5 were placed in a room in which the temperature was regulated to 37° C. ± 0.2°.

At temperature equilibrium, 15–20 minutes later, initial hematocrit determinations were made by the method of Parpart and Ballentine ('43). Enough solid NaCl or sucrose was added to make the plasma of Step 3 samples 1.5 or 2.5 or 3.5

times isotonic. After mixing, cell volume was measured in each by the hematocrit method and the volume-tonicity relations of this concentrated whole blood were plotted from the data obtained (fig. 2).

Step 7 The packed cells of Step 4 and the plasma-glucose solutions of Step 5 were rejoined and mixed well at zero time, and the resulting suspension was placed on a roller.

Step 8 At various times ca. 1 ml samples of the Step 7 suspension were centrifuged in the air turbine (Parpart and Green, '52). After separation about 0.4 ml of plasma was removed and the glucose content was determined by the method of Nelson ('44), using the Beckman spectrophotometer. Samples were read at 750 μ (Hempling, '54). At the same time hematocrit determinations were made and the changes in cell volume recorded.

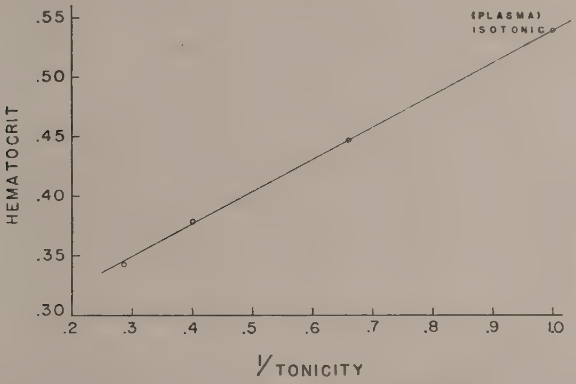

Fig. 2 Cell Volume-Tonicity Calibration. A calibration of the relation between red cell volume and the external osmotic pressure of the plasma, as measured by the hematocrit technique.

Thus by recording the disappearance of glucose from the plasma of these concentrated whole blood suspensions and the volume changes the cells are undergoing during the process, a precise measurement of the rate of penetration of glucose from the plasma into human red cells can be obtained. This method provides excellent environmental conditions for the erythrocyte. The cells remain in their own plasma. The glucose added is already dissolved in plasma, and the concentration range studied in these experiments (0.13–0.43 M)

is wide enough to obtain information about the mechanism of penetration without interfering with the ability of the cells to change volume. When exposed to extremely hypertonic solutions the red cell cannot be expected to behave as a normal osmometer. In order to obtain a high degree of accuracy, the time intervals for plasma-glucose samples recorded in experiments are the times measured from zero (mixing time for plasma glucose solution and cells) to the point at which the whole blood sample is being centrifuged at full speed in the air turbine.

Since separation of cells and plasma occurs so rapidly by this method of centrifugation (Parpart and Green, '51) it is certain that there would be very little glucose penetration from the plasma into the cells after the rotor head was at full speed.

RESULTS

Densimetry

Theoretical curves for the volume changes that erythrocytes should undergo with time in various glucose-saline solutions were calculated using an equation derived from Fick's Law by Schiødt ('33). This equation was selected for use in calculating the kinetics of the following experiments because it was derived specifically to describe the events that take place when erythrocytes are added to a hypertonic solution that contains a penetrating substance in the presence of a non-penetrating salt.

In Schiødt's final equation,

$$Kt = \frac{v_s - x}{v_o - x}\left\{(v_s - x) \quad \ln \frac{v_s - v_o}{v_s - v} + v_o - v\right\}$$

All volumes are expressed as per cent of that which the cells would have in normal plasma. v_s represents the final "plateau" or equilibrium volume of the cells, v_o the volume from which initial swelling begins, v the volume of the cells at any time (t), x the dispersed phase or osmotically inactive fraction of the cell, and K a relative diffusion constant which includes factors representing membrane thickness and surface area of the cell. The latter are assumed to be constant.

On inspection of the equation there appear to be 4 unknowns, v_s, v_o, x, and K. Schiødt assumes that there is no limitation to the application of the Boyle-Vant Hoff Law, $P_o (v_o - x) = P_1 (v_1 - x)$ where v_o and v_1 are volumes of cells in solutions of osmotic pressure P_o and P_1. This assumption was confirmed by Parpart and Shull ('35a) for the range of concentrations used in these experiments.

Applying the Boyle-Vant Hoff Law, v_s and v_o in the equation can be calculated if the osmotic pressure of the surrounding solution is known.

Schiødt derived empirically a value of 50% of the volume in plasma for the dispersed or non-osmotic fraction of the cell (x). In these experiments a lower value of 45% was used because it seemed nearer the true value for erythrocytes. However values of 50%, 45% or 35% make little difference in the final outcome of the calculations. Now if the diffusion constant, K, is known, then one will need only to substitute v in the equation and solve for the time, t, to that volume (v). Predicted cell volume changes with time could then be calculated for cells in solutions containing any combination of NaCl and glucose.

The value of the constant, K, was determined by trial and error. First, experimentally observed volume-time data for human red cells in various glucose-saline solutions obtained by the densimeter technique were plotted. Then by substitution in the equation a value for K of 0.16 was observed as that which would cause the theoretical curves to pass through the greatest number of experimental points.

This value of K remained constant for concentrations from 0.1 to 0.4 *M* glucose in 0.17 *M* NaCl-PO_4. Figures 3a and 3b show experimentally observed points plotted along the theoretical volume change curves for 0.1 *M* to 0.4 *M* glucose in 0.17 *M* NaCl-PO_4. The volume, v_o, from which initial swelling takes place, was calculated for each solution by the Boyle-Vant Hoff Law. The equilibrium or final volume, v_s, of the cells

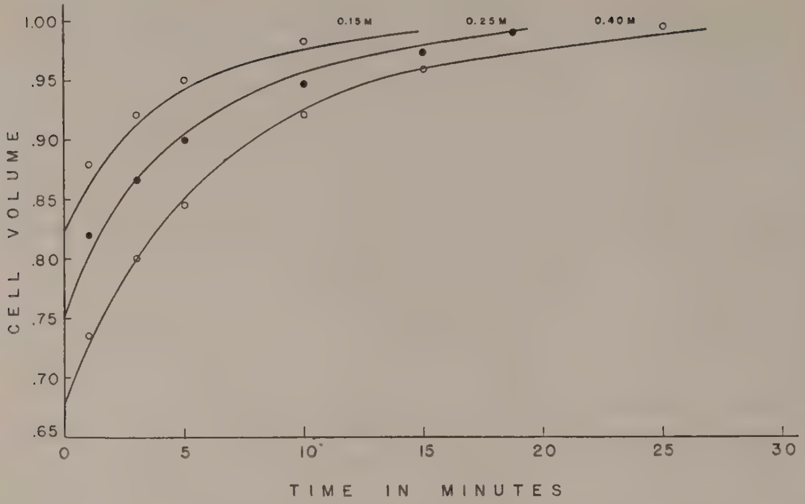

Fig. 3a Penetration of 0.1, 0.2 and 0.3 *M* glucose into human red cells. A comparison of experimentally observed volume changes (plotted points) to the theoretical volume changes (smooth curves) predicted for erythrocytes under these conditions by Ficks' Law (according to the Schiødt equation). Temperature 37° C., pH 7.78, K = 0.16.

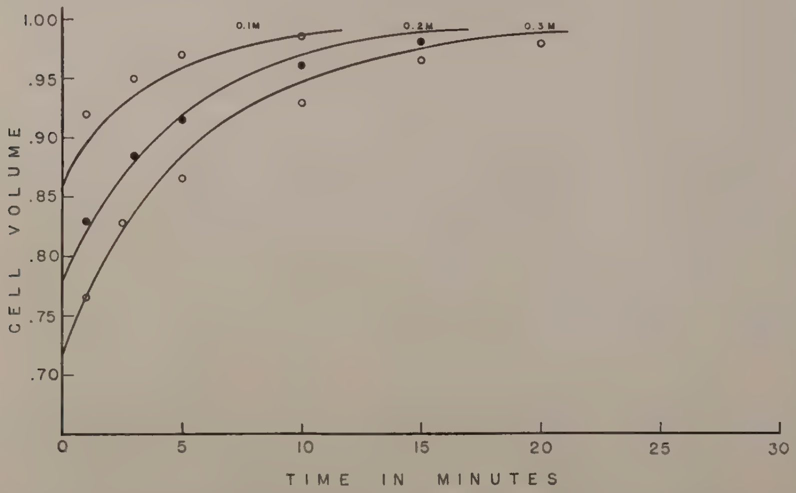

Fig. 3b Penetration of 0.15, 0.25 and 0.40 *M* glucose into human red cells. A comparison of experimentally observed volume changes (plotted points) to the theoretical volume changes (smooth curves) predicted for erythrocytes under these conditions by Fick's Law (according to the Schiødt equation). Temperature 37° C., pH 7.78, K = 0.16.

was taken as 100% of their volume in plasma in all experiments in which the glucose was made up in isotonic (0.17 *M*) NaCl-PO_4.

In all experiments of this type there seems to be no substantial difference between the volume changes predicted for the cells by the laws of simple diffusion and those volume changes which are experimentally observed.

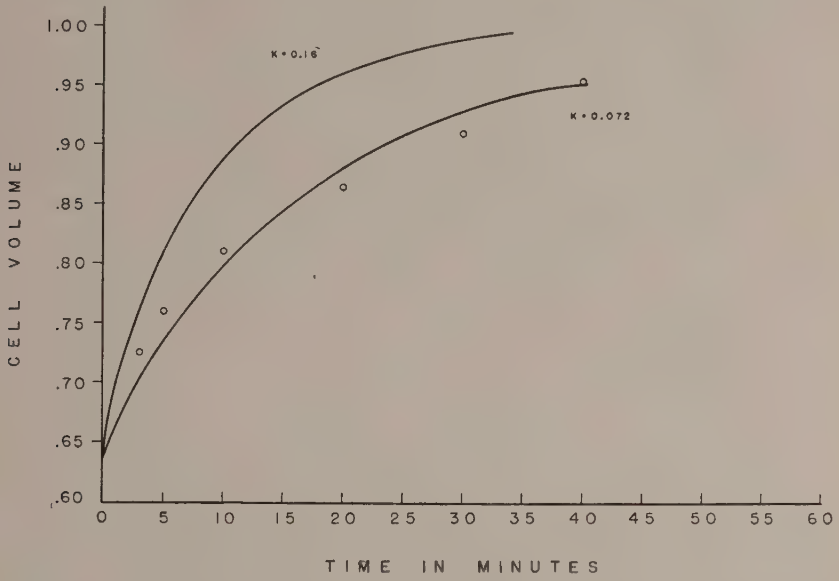

Fig. 4 Alteration in the relative permeability constant, K. When human red cells are exposed to 0.6 *M* glucose — 0.17 *M* NaCl-PO_4, the relative permeability constant, K, is radically altered. Experimentally observed volume changes (plotted points) are compared to the theoretical volume changes (smooth curves) predicted by Fick's Law when values of $K = 0.16$ or $K = 0.072$ are used in the Schiødt equation. The pH is 7.78, temperature 37° C.

Only after addition of cells to extremely concentrated solutions of glucose in 0.17 *M* NaCl-PO_4 is the permeability constant radically altered. Figure 4 shows the volume changes which cells undergo in a solution of 0.6 *M* glucose in 0.17 *M* NaCl, compared with the predicted volume changes for such a situation. If the K value is reduced by approximately one-half, the experimental results and the theoretical curve coincide.

As a further test, erythrocytes were added to solutions containing the same varying concentrations of glucose as in previous experiments (0.1 *M* to 0.3 *M*) but prepared with NaCl-PO_4 (see methods, page 181) so that the osmotic pressure of each solution was approximately two times isotonicity. Using this procedure, the cells in all suspensions are subjected to the same amount of shrinkage before swelling begins. It is then possible to determine whether shrinkage, over the concentration range used in these experiments, will radically affect the physical state of the hemoglobin in the cell (Ponder, '44, '45) or the structure of the cell membrane, in such a way as to alter the rate of penetration of glucose into the cell from solutions of high or low glucose concentration.

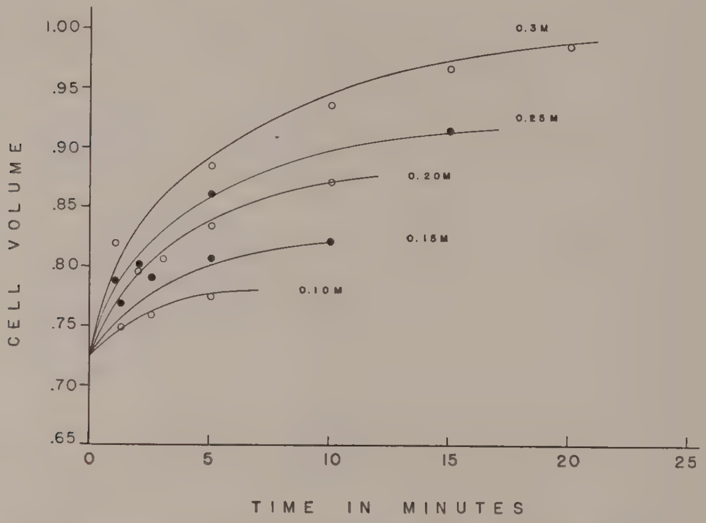

Fig. 5 Penetration of 0.1, 0.15, 0.20, 0.25 and 0.30 *M* glucose from NaCl—PO_4 solutions with osmotic concentrations equivalent to two times isotonicity. Experimentally observed volume changes (plotted points) compared to theoretical volume changes (smooth curves) pH 7.7, temperature 37° C., K = 0.16.

Figure 5 gives the results of a series of experiments of this type and compares these to the theoretical curves calculated for the volume changes the cells should undergo under these new conditions. The volume of the cells from which initial swelling begins, v_o, as calculated by the Boyle-Vant Hoff Law for solutions with an osmotic pressure of two times isotonicity, was found to be 72% of their volume in plasma or isotonic saline. Since there is much experimental evidence for the assumption that cells in all these solutions of two times isotonicity will undergo the same amount of shrinkage before swelling begins, this value for v_o is constant for cells in all the solutions of this series. The equilibrium volume, v_s, of the cells varies in inverse proportion to the osmotic pressure of the non-penetrating salt in the solution used. This value was calculated from the concentrations of the NaCl employed, by the Boyle-Vant Hoff Law.

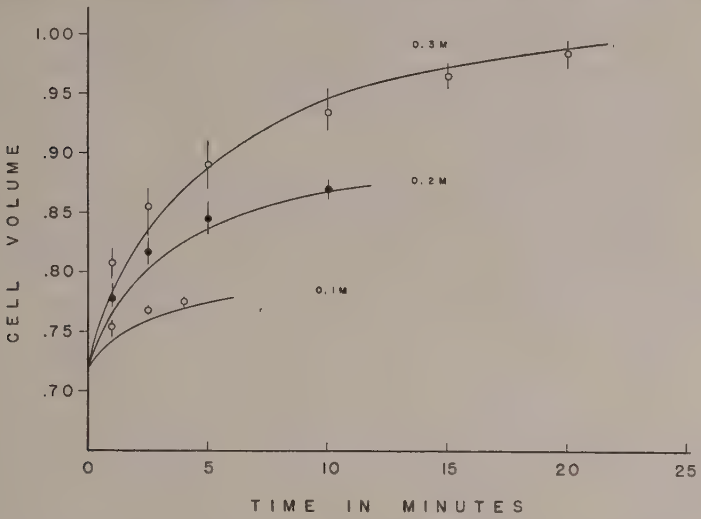

Fig. 6 The maximum, minimum and average values of experimental points from eight experiments are compared to the predicted volume changes (smooth curves) for red cells in solutions containing 0.1, 0.2 and 0.3 *M* glucose made up in NaCl-PO_4 to osmotic concentrations of two times isotonicity. The pH is 7.78, temperature 37° C. and relative permeability constant, K = 0.16.

│ — maximum
○ — average
│ — minimum

Figure 6 presents the spread of the results of 8 experiments with 0.1 M, 0.2 M and 0.3 M glucose in solutions at the osmotic pressure of twice isotonicity. The accompanying curves are calculated from the Schiødt equation. Once again, using the K value of 0.16, the calculated volume changes fit the experimentally observed points. This value remains constant throughout the range of concentrations tested. There is no observable effect of previous shrinkage of cells on the rate of glucose penetration at least up to a concentration producing double the normal osmotic pressure. More important, there is no evidence of any radical deviation in the permeability of these cells to glucose from the permeability predictable from the laws of simple diffusion.

The results of these densimetric experiments show that the same permeability constant for glucose penetration into human red blood cells can be calculated by two different experimental approaches, and that this constant does not change over a wide range of glucose concentrations.

Chemical analytical results

Figure 7 represents a typical experiment illustrating both the alterations in red cell volume and the disappearance of glucose from the plasma of slightly concentrated whole blood (hematocrit: 55% cells). The resulting shrinkage-swelling curves are typical of experiments performed in this manner, Parpart and Shull ('35a). Equilibrium between the cells and their environment is reached after 30 minutes, 60 minutes and 120 minutes respectively in 0.14 M, 0.29 M and 0.43 M glucose.

It is possible to calculate a relative permeability constant for these reactions by the use of an equation derived for these experimental-conditions for Fick's Law by M. H. Jacobs.[3] In these experiments the volume of plasma relative to red cell volume is nearly equal, hence the external glucose concentration can no longer be considered as unchanging, as it was in Schiødt's equation, though the latter applied to and fit the densimeter results. Under the present condtions when the cells shrink upon mixing with the plasma-glucose solution, the external environment is diluted considerably. The concentration of the glucose in the external environment undergoes further changes as the glucose penetrates the cells. Jacobs derived the equation in the following manner: the unit of volume is taken as the cc; the unit of quantity for each solute as the amount that, dissolved in 1 cc of water, will

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[3] See "Acknowledgments."

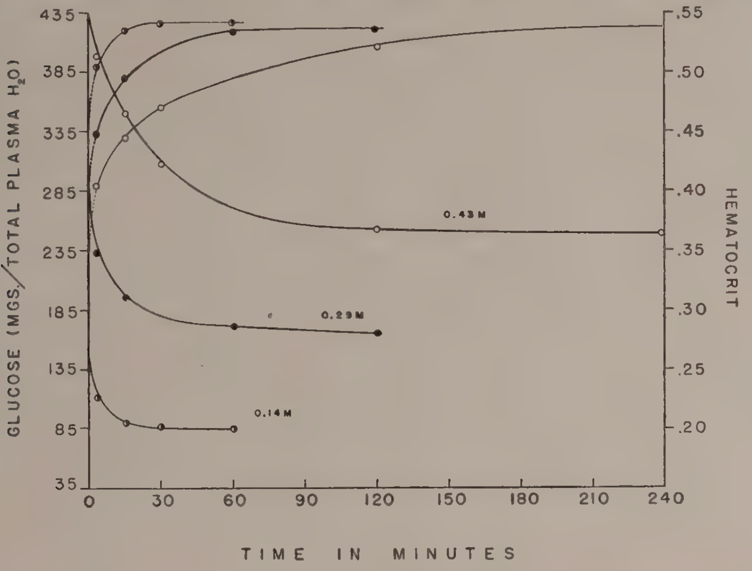

Fig. 7 After the addition of 434, 288 and 144 mg of glucose to the plasma of three 7 cc samples of slightly concentrated whole blood (hematocrit: 54.6%) resulting in concentration of 0.43, 0.29 and 0.14 *M* glucose respectively, the disappearance of glucose from the plasma and the cell volume changes which accompany the penetration of this solute (glucose) have been measured and recorded.

Dotted lines connect points representing the theoretical minimum volume (from which swelling begins) and the first experimentally observed volume. Minimum volumes were calculated from the volume tonicity calibration — Figure 2.

give a solution isosmotic with normal plasma and erythrocytes. For glucose the unit of quantity is therefore 54 mg (osmolar concentration 0.3 M) and a solution having this amount of glucose in 1 cc will have a concentration of unity. All concentrations are thus measured as fractions of the isotonic unit.

For theoretical treatment consider a volume of blood containing exactly 1 cc of water in cells and plasma together. In actual experiments the volume of blood water used will in general not have this value but some other value, w. But by dividing the actual volume of blood water and also the amount of each of the solutes contained in it by w, the problem is reduced to the form chosen for treatment.

Of the 1 cc of water contained in the actual or theoretical sample of blood a fraction (and also in this case a volume in cc), V, will be contained in the cells and a fraction (and volume), 1-V, in the plasma. Regardless of shifts of water between cells and plasma the total amount in the system will remain constant and equal to unity.

Dissolved in the volume V and 1-V respectively of the blood water, are quantities a and b of osmotically active, non-penetrating solutes. Because these solutes cannot cross the cell boundary a and b must remain constant, although in the present situation the *concentrations* of these solutes will change with changes in V and 1-V.

In normal blood at the beginning of an experiment we have isotonicity of cells and plasma and therefore

$$\frac{a}{V_o} = \frac{b}{1 - V_o} = 1 \tag{1}$$

from which it follows that $a + b = 1$. Since a and b do not change, their sum must continue to be unity regardless of the value of V.

Now add to each cc of the blood water, Q units of a penetrating solute which enters the cells slowly as compared with water, so that a condition of osmotic balance between the cells and the plasma is maintained at all times. For osmotic equality, we have

$$\frac{a + s}{V} = \frac{b + Q - s}{1 - V} \tag{2}$$

solving for s

$$s = (a + b + Q)\, V - a = (1 + Q)\, V - a \tag{3}$$

$$d\, s = (1 + Q)\, d\, V \tag{4}$$

Immediately on the addition of the solute there will be an outward movement of water to equalize the osmotic pressure.

On the assumption that up to this time S remains virtually equal to zero, equation (1) shows that the volume has decreased to $\frac{a}{a+b+Q}$ which may be called $\overline{V}_o$ and is the volume assumed to exist at the time $t=0$ when the solute begins to enter the cells. Obviously $\overline{V}_o$ is smaller than V_o, which is shown by equation (1) to have the value $\frac{a}{a+b}$. From the minimum volume V_o, V now increases until equilibrium, V_{eq} is reached. At this volume the osmotic pressure inside and outside the erythrocytes is equal (Equation 2) and diffusion equilibrium of the solute $\frac{s}{V}=\frac{Q-S}{1-V}$ also exists. By subtracting this relation from equation (2) it follows that at equilibrium $\frac{a}{V_{eq}}=\frac{b}{1-V_{eq}}$. Comparison of this result with equation (1) shows that $V_{eq}=V_o$.

The kinetics of the swelling process between the volumes $\overline{V}_o$ and V_o is assumed to obey Fick's Law,

$$\frac{ds}{dt}=PA\left(\frac{Q-S}{1-V}-\frac{S}{V}\right) \tag{5}$$

where P is the usual permeability constant and A the area in cm^2 across which diffusion occurs. Combining equations (4), and (5)

$$PAt=(1+Q)\int\frac{(V-V^2)}{a-V}\,dV=(1+Q)\int\frac{V\,dV}{a-V}-(1+Q)\int\frac{V^2\,dV}{a-V} \tag{6}$$

Performing the integration and noting that when $t=0$, $V=\overline{V}_o$, we have

$$PAt=(Q+1)\Big[(a-a^2)\ \ln\frac{a-\overline{V}_o}{a-V}-(1-2a)\ (V-\overline{V}_o)$$
$$-\frac{1}{2}\Big\{(a-\overline{V}_o)^2-(a-V)^2\Big\}\Big] \tag{7}$$

The equation in this form may be used either with volumes of cell water obtained from hematocrits using the equation $V=Hct\text{-}(1\text{-}Hct)\ x$, when x is the non-solvent volume of the erythrocyte whose water volume is V, or with such volumes as can be calculated from chemical analysis by the relation

$$V=\frac{a+S}{a+b+Q}$$

Thus it was possible in these experiments to determine relative permeability constants by two different methods of calculation, for the hematocrit value was measured at the same time that samples were taken for glucose determinations (S value).

Tables 1A and 1B show PA, or K (relative permeability constant) values determined for the rate of penetration of various concentrations of glucose. The K values in table 1A were obtained by calculating the volume of cell water (V) from hematocrit values at different times (3 minutes and 15 minutes), and substituting in equation (7) above. The K values in table 1B were determined by the use of glucose analysis data.

TABLE 1A

Relative permeability constants, K, calculated on basis of hematocrit data

Glucose isotonic unit	.48 –51			I	1.43 – 1.55	
Glucose conc. M/L	.13 *M*	.14 *M*	.15 *M*	.29 *M*	.4 *M*	.43 *M*
3 Min. K =	.092	.13 .10	.09	.073 .069	.047	.05 .045
15 Min. K =	$V=V_0$	$V=V_0$		.035 .02	.013	.028

TABLE 1B

Relative permeability constants, K, calculated on basis of glucose analyses

Glucose isotonic unit	.48 – 51			I	1.43 – 1.55	
Glucose conc. M/L	.13 *M*	.14 *M*	.15 *M*	.29 *M*	.4 *M*	.43 *M*
3 Min. K =	.072	.09 .081	.073	.087 .053	.04	.033 .044
15 Min. K =	$V = V_0$	.06 .055	$V = V_0$	.044 .027	.02	.028 .021

In these calculations the expression $V = \frac{a + S}{a + b + Q}$ was employed in obtaining the volume of cell water (V) at different times. It should be made clear that the volume (V), as calculated in this manner is also dependent upon the hematocrit measurements. The amount of glucose that has entered the cells (S) in the equation $V = \frac{a+S}{a+b+Q}$ can be obtained only when the relative volumes of cells and plasma are known. S (the amount of glucose that has entered the cells) was always expressed isotonic units. All calculations for tables 1A and and 1B were made with the assumption that human red blood cells contain about 55% (osmotically active) H_2O, (as in the densimeter experiments). Thus, in these cases, the per cent water in the whole blood used was calculated to be of the order of 73–75%.

TABLE 2A

Relative permeability constant, K, calculated on the basis of hematocrit data

Glucose isotonic unit	.48 – 51			I	1.43 – 1.55	
Glucose conc. M/L	.13 *M*	.14 *M*	.15 *M*	.29 *M*	.4 *M*	.43 *M*
3 Min. K =	.11	.14 .11	.12	.11 .076	.066	.081 .070
15 Min. K =	$V=V_0$	$V=V_0$	$V=V_0$	.043 .03	.022	.054

TABLE 2B

Relative permeability constant, K, calculated on basis of glucose analysis

Glucose isotonic unit	.48 – 51			I	1.43 – 1.55	
Glucose conc. M/L	.13 *M*	.14 *M*	.15 *M*	.29 *M*	.4 *M*	.43 *M*
3 Min. K =	.094	.08 .092	.066	.062 .05	.066	.04 .032
15 Min. K =				.04 .023	.022	

Since it is well established that human red blood cells contain a total water of ca 65%, it was decided to re-calculate the constant in the same manner using 65% as the total water content of the red cells. In these cases the per cent water of the whole blood used was calculated to be of the order of 79–81%. Tables 2A and 2B show the K values determined under these conditions.

Upon inspection of the tables, it is clear that the value assumed for percentage of H_2O in the cells makes little difference to the K values obtained. This is in agreement with densimeter results. Also, comparison of the values in the tables show that the "relative permeability constants" calculated on the basis of hematocrit data alone are slightly higher than those calculated by the use of glucose analysis data. This is not surprising, since the determination of "K" from the glucose data involves a greater number of steps both in the calculation and in the experimental procedure. Thus a more variable K might be expected.

Of particular interest, however, is the difficulty in obtaining a "K" value which will remain constant throughout an experiment. It has been reported many times that the peremeability constant for glucose penetration into human red cells decreases with time (Bjering, '32; Meldahl, Ørskov, '40). The results of these experiments involving chemical analytical data would seem to substantiate this. However, it should be pointed out that the decrease of the K value with time found in these experiments is not a phenomenon peculiar to the higher concentrations of glucose or to any one particular concentration of glucose. But, rather, a decrease in the K value can be obtained at any experimental concentration if the measurements are made when V (volume of cell water) is near to V_o (the initial volume of cell water).

If the measurements are made in the early part of the reaction (when V is much less than V_o, and thus the changes taking place most pronounced) it is possible to obtain a permeability constant that is practically the same for any concentration of glucose. This suggests that perhaps the decreasing "permeability constant" as the reaction proceeds is more a function of difficulties in obtaining accurate measurements when the changes taking place in the experimental system are small, rather than an expression of an alteration in the rate of penetration as the glucose enters the cells. It is significant to note that the concentration of glucose in the plasma can be doubled from 0.5 Isotonic Units (0.13-0.15 M glucose) to 1.0 Isotonic Unit (0.29 M glucose) without a significant change in the K value. Only at concentrations of 1.5 Isotonic Units (0.4-0.43 M glucose) is there a slight decrease in the values of the constant. Furthermore, these

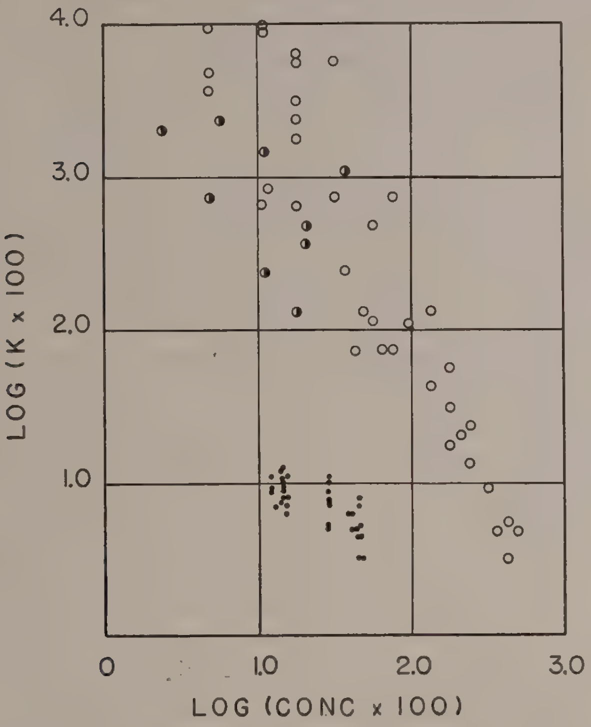

Fig. 8 Comparison of relative permeability constants (K), obtained in this investigation with those reported by Wilbrandt et al., 1947.

○ ◑ Wilbrandt's K values
○ — obtained by indirect osmotic technique
◑ — obtained by direct osmotic technique
● K values obtained in present investigation
● — chemical analysis

K values certainly do not show the great alteration with varying concentration that have been reported by Wilbrandt ('47). Figure 8 shows the calculated K values obtained for the glucose penetrations at the concentrations used in these experiments, and is compared with K values otbained by Wilbrandt et al. ('47). Using indirect osmotic methods, Wilbrandt found that by increasing the external concentration of glucose 100 times (0.015 M–1.5 M) the constant may change by a factor of one thousand.

Calculation of the absolute permeability constant [4]

The permeability constant P in most common use at present has the dimensions of centimeters/hour and tells how many units of quantity will cross 1 cm^2 of cell surface in one hour with a difference in concentration across the cell membrane of one unit of quantity per cubic centimeter. The thickness of the cell membrane (unknown for most cells) is not used in finding P, as it is for example in determining the diffusion coefficient D, which has the dimensions of square centimeter/hour.

To convert the relative constant K (which is here used merely as a convenient test of the applicability of Fick's Law) into the true permeability constant P, a change must be made in some or all the units of time, area, and volume. The unit of quantity used is without effect on the values of K and P, provided that the same unit is used to measure both amount of diffusion and concentration; no change in it is therefore required.

In Schiødt's equation for finding K time is measured in minutes; to change the unit to hours, K must be multiplied by 60. Furthermore, Schiødt's V's represent not primary volume in cubic centimeters but rather ratios of actual volumes to an initial cell volume, which for the human erythrocyte may be taken as 90×10^{-12} cm^3. If therefore all V and V — X terms in Schiødt's equation be multiplied by 90×10^{-12} cm^3

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[4] See footnote 3, page 190.

they will become volumes in cubic centimeter, as they should be for determining P as defined above. Inspection of the equation shows that this procedure has the effect of multiplying K by 90×10^{-12}.

In Schiødt's equation a term for area does not appear, as it presumably should in the description of any process governed by Fick's Law. However an area term, A, may be considered to be present but invisible because it has the value of unity. In other words Schiødt, in effect takes his unit of area to be that of the surface of a single erythrocyte, which according to Ponder ('48) for those of man, is 163×10^{-8} cm². The last step, therefore, needed to convert K into P is to divide the former by 163×10^{-8}. Summarizing the procedure

$$P = K \times \frac{90 \times 10^{-12}}{163 \times 10^{-8}} \times 60 = K \times 33 \times 10^{-4}$$

For the value of $K = 0.16$ used for the construction of the curves $P = 5.3 \times 10^{-4}$ cm/hour.

In the experiments made by chemical analytic methods, the equation of Jacobs also provides a relative constant K for testing the applicability of Fick's Law. It should be noted, however, that this K is not the same as Schiødt's K and that the agreement here found in the order of magnitude of the two is purely accidental. On the other hand the even better agreement of P in the two cases, which will now be shown to exist, is truly significant, since P, as defined above, always has the same meaning. For all comparisons, therefore, P rather than K must be used.

The relation of the second K to P as previously stated is $PA = K$, or $P = K/A$, where A is the area in cubic centimeters through which the observed diffusion occurs. It will be noted that in the derivation of equation (7) the unit of volume was so chosen that all the V's in the equation are measured in cubic centimeters as they should be to find P directly, and that all concentrations are measured in amounts per cubic centimeter of water. The area, A, through which diffusion occurs must be taken as the total area of all the erythrocytes associated with 1 cm^3 of blood water. The value of A for a typical case can be calculated as follows:

Hematocrit	*55%* (see legend for figure 7)
Volume per cent of effective cell water	*65%*
Volume per cent of plasma water	*92%*
Calculated volume of blood water	*77%*
Volume of blood containing 1 cm^3 of water	*1.30* cm^3 $(1 \div 0.77)$
Volume of erythrocytes associated with 1 cm^3 of blood water	*0.715* cm^3 (1.30×0.55)
Number of erythrocytes	8×10^9 $(0.715 \div (90 \times 10^{-12})$
Total surface of erythrocytes	*13,000* cm^2 $(8 \times 10^9 \times 163 \times 10^{-8})$

We now have $P = K \times 60 \times \frac{1}{13{,}000} = K \times 46 \times 10^{-4}$

In table 2A for which the effective cell water was taken as 65%, the average value of K for three minutes and for concentrations 0.13 to 0.15 M inclusive (0.5 Isotonic units) was 0.12. Multiplying this value by 46×10^{-4}, we have $P = 5.5 \times 10^{-4}$ cm/ hour which is almost identical with the value 5.3×10^{-4} obtained by the densimeter technique. For a concentration of 0.29 M glucose (one Isotonic unit) using the average value of $K = .09$ from the same table, $P = 4.2 \times 10^{-4}$.

If, to be more comparable to densimeter values the effective cell water be taken as 55% the factor by which K is multiplied to obtain P becomes 43×10^{-4}. Using the average K for the time and concentration in question from table 1A, 0.10 for concentrations in the range of .5 Isotonic units (0.13 M to 0.15 M) and 0.7 for concentration of one Isotonic (.29 M) unit $P = 4.3 \times 10^{-4}$ in the former and $P = 3.0 \times 10^{-4}$ in the latter case.

For higher concentrations and longer times, and for the chemical method in general the P values are somewhat smaller than those just calculated.

DISCUSSION

The results of these experiments indicate that the mechanism of glucose penetration into human red blood cells may not be as complex as previously reported. However, the question of the existing conflict of data in the literature concerning the behavior of human erythrocytes when exposed to glucose in varying concentrations immediately presents itself. Perhaps the problem may be resolved by an analysis of the techniques employed in the present and previous investigation of this type.

In the experiments reported in this paper the erythrocytes were kept in their normal plasma and never stored. It is not clear what changes take place when red cells are removed from their plasma and washed in isotonic saline but it has been demonstrated that changes do occur. Bjering ('32) reported that penetration of glucose occurred much more rapidly when cells were in plasma than in one per cent NaCl. This environmental effect was also demonstrated by Klinghoffer ('40) when he found that by adding *dry glucose* to whole blood much more rapid rates of penetration and equilibrium distribution were observed than those found when glucose *in solution* was added to whole blood. Whether these changes in cell behavior involve a decrease in the internal osmotic pressure of the cells and subsequent increased cell fragility (such as occurs on storage in isotonic NaCl solutions) or whether they are a manifestation of a direct effect on the cell membrane is a difficult question to answer. The problem is certainly worthy of further study, and until it is resolved these differences should be kept in mind when analyzing or comparing results of experiments of the type presented here.

Permeability characteristics of red blood cells are also altered by storage. Schiødt ('31) and Bang and Ørskov ('37) reported that the cells from fresh blood are much more permeable than those of blood that has been stored. Meldahl and Ørskov ('40) found permeability constants for red cells decreased with time of storage of the blood.

In the experiments described in this paper, the freshness of the blood sample considerably affected the results obtained. In order to obtain duplication of results, blood samples were not used for experiments when they had been out of the individual for more than 4 hours.

LeFevre ('48, '50, '52) has performed numerous experiments by a densimetric technique on the rate of penetration of glucose into human red cells. He has reported storing cells in NaCl solutions for periods up to 10 days without noticing a change in the permeability characteristics of the cells, although no experiments were actually performed to test the effect of storage itself. It seems probable that such treatment could result in an alteration of the ability of the cells to change their volume (Parpart et al., '47). In many experiments he has exposed and equilibrated cells in saline at 60% of isotonicity before adding glucose. The reasons are most probably that the optical effects of the volume changes show more clearly and thus smoother records are obtained. From the point of view of maintaining the red blood cells as normal osmometers, this technique is open to criticism. Jacobs and Parpart ('33) have demonstrated that pre-exposure of red cells of man to slightly hypotonic solutions of NaCl or sucrose will markedly affect their osmotic resistance. Hence, if one is primarily interested in rates of volume change such experimental conditions must be avoided.

One more point should be mentioned in regard to densimetric technique. In the present experiments, the cells in plasma were added to 7 cc of glucose-saline solution. They first shrink to a volume determined by the osmotic concentration of the solution and then swell as the glucose penetrates. Following this procedure one can be certain that all the cells concerned are subjected to the same degree of initial osmotic pressure increase (osmotic shock). And in the range of concentrations employed, the osmotic pressure increase should not have been so great as to cause any of the cells to manifest subsequent abnormal volume changes. LeFevre, however, equilibrated cells in 5 or 10 cc of saline solution and then added 0.5 or 1 cc of a highly concentrated glucose solution (usually 6 times the final concentration desired). The suspension was well stirred at the time of addition, but the possibility exists that a number of cells in suspension were exposed to an osmotic pressure increase from which they did not recover; that is, their ability to swell again may have been greatly altered by an "osmotic shock." Ponder ('44), reporting on the osmotic behavior of crenated rat red cells, suggests that the problem of the anomalous slight swelling of red cells under certain conditions may be explained (although not completely solved in a quantitative sense) by assuming that crenation of red cells may be synonomous with a gelation or paracrystalline state of the Hb molecule in the cell interior. It is probable that sudden exposure to an extremely hypertonic glucose solution may bring about this change in the physical state of hemoglobin (Jacobs, Parpart, Glassman, '38). The gelation or paracrystalline state is characterized by a great increase in the osmotic resistance of the cells (Ponder, '45). Under these conditions their volume changes would be unrelated to the Boyle-Vant Hoff Law. Such an effect would cause a marked discrepancy between experimentally observed and predicted volume changes.

Widdas ('54) has used much the same procedure for densimetry as LeFevre. In the Widdas system 21 cc of a highly diluted whole blood — one per cent NaCl suspension is placed in a chamber and injections of hypertonic glucose solutions (1.7 M) are added to bring about a desired final glucose concentration.

The injected quantities range from 0.5 ml to 5 ml of 1.7 M glucose. However it is pointed out that satisfactory results are only obtained with injections from 0.5 to 2.0 ml. Such injections would bring about a final concentration of approximately 0.03 to 0.15 M glucose in one per cent NaCl. Over this concentration range Widdas finds that the permeability constant for glucose changes by a factor of fifteen. The results dealing with glucose concentrations above this range are not presented in his report. Widdas then applies his results to a kinetic system for a "facilitated" transport of glucose into the cell which involves a near-saturated carrier; and his data for the 4 experimental concentrations reported fall on a straight line, the slope of which represents a constant for the carrier. It should be pointed out that Widdas mentions that with shorter periods of washing of cells with NaCl, the value for this carrier constant is shifted. He stresses that the influence of the preparation of the suspension on the results obtained should be studied before detailed conclusions can be drawn. Widdas reports no concentration at which his transport system fails to operate.

Once again the techniques employed may be questioned. Under the conditions of the experiments a sample of whole blood is diluted 1 part in 5000 parts NaCl. Twenty-one cubic centimeters of this very dilute cell suspension is placed in a chamber and varying amounts of hypertonic glucose solution is injected into the system. In experiments dealing with high final glucose concentration (0.3 M) this procedure would involve injections of 5 ml of 1.7 M glucose. The effects of this treatment on the cells was not reported, but in our own experience a considerable number of cells in the suspension are crenated or osmotically shocked in the process. Such a result would cause a great difference in the experimental and theoretical volume change curves.

The experiments that have been reported dealing with low final glucose concentrations (0.3 to 0.15 M) involve very small volume changes. This would make the measurement of differences in rates of penetration between cell suspensions over this concentration range a very difficult problem. Any source of error would be greatly magnified in the calculations. The problem is further complicated by the fact that final concentrations in the experiments reported were attained by repeated injections of 0.5 cc of 1.7 M glucose into the same dilute cell suspension. With each injection a new dilution effect must be calculated, and another possibility is introduced of altering by "hyper-osmotic shock" the ability of some of the cells to change volume. Considering the small volume changes being measured and the relatively few cells present, these sources of error might seriously influence the apparent rate of volume change in these experiments. This in turn could account for the wide range of "permeability constants" reported by Widdas ('54).

The use of metabolic inhibitors in volume change experiments with glucose and human red cells presents another problem. Many of these inhibitors show little or no effect on the volume changes which red cells undergo in glucose-saline solutions. LeFevre ('48) reports that iodoacetate, copper, alloxan, mapharsen, arsenite and lead are ineffective; while phlorizin, a substance that has been found to be a very specific inhibitor of glucose transport mechanism in kidneys and intestine, shows very little effect on glucose penetration in human red cells. LeFevre reports 1_2, Hg^+, Hg^{++} and parachlormercuribenzoate as the most effective inhibitors employed. Since there seems to be no specific inhibitor in the group, how are these results to be interpreted? It is interesting that when low concentrations of these substances are added to human red cells suspended in saline, a subsequent injection of a concentrated glucose solution will not bring about the predicted volume changes of the red cells. But can this effect be localized and attributed to the blocking of an active carrier mechanism for glucose? LeFevre ('48) found the same effects when glycerol is used as the penetrant and concludes that both these non-electrolytes enter by an active process.

Parpart, Barron and Dey ('47) have shown that parachloromercuribenzoate retards the apparent rate of water penetration into human red cells. They suggest that this substance may be affecting the outward diffusion of hemoglobin. Riggs ('52) has reported that this same substance lowers the oxygen tension of Hb in solution. It is known that an alteration in oxygen tension will affect the equilibrium degree of swelling of red cells (Jacobs and Parpart, '31).

It seems logical that a substance should be proved to have no effect on the internal osmotic pressure of red blood cells or their cell membranes before investigating its effect on penetration of a substance by a volume change technique; and in the case of most inhibitors employed, such experiments have not been performed.

Another interesting approach to the problem of glucose penetration involves the study of comparative kinetics and competition between various sugar molecules. LeFevre and Davies ('51), report that the aldose sugars, galactose, xylose, arabinose, mannose and dextrose all penetrate by participation in an active process at the cell surface. In their experiments dealing with the ketose sugars, sorbose and levulose, no such process is evident and the laws of simple diffusion are obeyed; that is, the cells swell progressively faster with increased external sugar concentrations.

These results indicate that there should be complete independence between the movement of an aldose or a ketose occurring together in a red cell suspension. Similarly there might also be a competition for the carrier mechanism of the cells, between two aldoses occurring together in a red cell suspension.

Wilbrandt ('47) reports that xylose, arabinose and glucose do compete with each other in suspensions of red cells, and that the total rate of penetration was practically the same as from solutions of the components alone, although the concentration was 3 × higher. LeFevre and Davies ('51) find that this is not the case in their experiments. The rate of swelling is always less than would be expected by an additive treatment of the curves for the separate entry of each sugar. The results of such experiments are difficult to interpret. However, they do not clearly indicate two separate processes for the entrance of aldoses and ketoses into red cells. Parachloromercuribenzoate and Hg^{++}, the inhibitors LeFevre employs, affect penetration of aldoses and ketoses in the same way.

Jacobs ('54) reports a similar "competition" between ethylene glycol and glycerol for entrance into the human red cell and states that the mechanism of this effect is unknown.

There is much to be learned about the osmotic volume changes which red cells undergo in complex solutions. But whenever a competitive effect is found between non-electrolytes in suspensions of red cells, it cannot be safely concluded that a specific carrier for that type of molecule exists on the cell surface.

If the mechanism for the penetration of any molecule is to to be clearly elucidated it is necessary that the experiments should be as simple and direct as possible, and the cells should not be exposed to any treatment that may alter their ability to exhibit normal osmotic volume changes. This was the plan of the experiments reported here, and their results are incompatible with any of the recent theories advanced for the mechanism of glucose penetration into human red cells.

The best interpretation of the data obtained under the experimental conditions here described is that glucose enters human erythrocytes by a simple diffusion process.

The relative permeability constant calculated from the data obtained with the photoelectric densimeter does not change over a wide range of glucose concentrations (0.1 M–0.4 M in 0.17 M NaCl). Theoretical volume change curves calculated by the Schiødt equation for simple diffusion under these exact experimental conditions, invariably fit into the experimentally observed volume change data when a K value of 0.16 is used.

Altering the experimental technique and subjecting red cells to moderate decrease in volume did not change this relative permeability constant, or affect the agreement of the predicted and experimental volume change curves. The rate of penetration or swelling was still determined by the concentration of glucose in the external environment; the higher the glucose concentration, the greater the amount of glucose that enters the cell per unit time.

The data obtained by chemical analytical procedures corroborate the passive diffusion interpretation offered above. The results show that the external concentration of glucose (in the plasma) can be doubled from 0.14 to 0.29 M without markedly affecting the relative K values.

Figure 8 presents clearly the results of the chemical analytical experiments and shows no significant variations of relative K values in comparison to those previously reported by Wilbrandt et al. ('47).

The reasons for Wilbrandt's choosing such a range of glucose concentrations are not clear. Surely when the external concentration of glucose is above 0.6 M, the cells in suspension will no longer behave as normal osmometers. It is a pointless procedure to further increase the hypertonicity of external medium from that point (0.6 M) to 1.5 M glucose, and to calculate permeability constants from whatever volume changes that may occur. Information resulting from experiments in which red cells are subjected to such extraordinarily hypertonic conditions cannot possibly confirm or disprove any theory concerning the penetration of glucose into the normal erythrocyte.

Further, on considering Wilbrandt's experiments with dilute concentrations of glucose, it should be pointed out that the volume changes cells undergo in suspension in which the external glucose concentration ranges from 0.015 to 0.05 are very minute and rapidly concluded. Measurements under such conditions would be extremely difficult and the slightest error would be greatly magnified.

Most importantly, the high variability of Wilbrandt's K values over the concentration range of 0.11 M to 0.3 M glucose should be noted. At some concentrations, different experiments give K's that vary by a factor of 100. No such variation is evident in results of the present investigation. For penetration studies of this type, the concentration range of 0.1 M–0.3 M is most suitable. Over this range the extent of the volume changes the cells undergo and the time course of the reactions are conducive to accurate measurements. It is also possible to double or triple the external concentration of the penetrant without altering the ability of the cells to undergo normal volume changes.

The fact that Wilbrandt's K's are so variable over this range suggests that the techniques employed in his investigations are unreliable.

Consideration of the results of this investigation makes it clear that there is no reason to postulate an active mechanism for glucose penetration into human red cells.

No evidence has ever been presented for the uptake of glucose by the cells against a concentration gradient; the apparent evidence for an active transport process has been based upon the failure of the human red cells to exhibit volume changes predicted by Fick's Law for simple diffusion, and upon the wide variation in permeability constants that have been calculated by substituting such volume change data in diffusion equations derived from Fick's Law. However, an attempt has been made in this discussion to point out several experimental factors that may have altered the ability of the cells to exhibit normal volume changes.

It has been shown in these investigations that if human red cells are used immediately after withdrawal from the donor, without separation from their plasma, and not subjected to any excessive hyperosmotic pressure increase, glucose will penetrate with ease from external concentrations of as high as 0.4 M. The volume changes of human red cells under the experimental conditions outlined in this paper has always been predictable and in agreement with Fick's Law of simple diffusion.

Absolute permeability constants (P) have been calculated from the relative constants obtained by desimeter and chemical analytical technique so that the results of these experiments may be properly compared to each other and to the permeability constants for other substances and other cells that have been calculated in the past (Jacobs, '52). The values obtained show no variation that would suggest a mechanism more complicated than simple diffusion for the penetration of this molecule into the human red cell.

SUMMARY

1. The rate of penetration of glucose into human erythrocytes has been studied by a densimeter technique and by a method of direct chemical analysis.

2. A photoelectric densimeter was used to measure the volume changes which erythrocytes undergo in glucose-saline solutions of varying concentrations. These were compared to theoretical volume change curves predicted by a diffusion equation derived from Fick's Law by Schiødt.

3. Glucose, at concentrations from 0.1 *M* to 0.4 *M* in 0.17 *M* NaCl-PO_4 at pH 7.78, 37° C., enters the human red cell with ease, and the volume changes which accompany the penetration always coincide with the volume changes predicted by Fick's Law.

4. The relative permeability constant (K) for these experiments is found to be 0.16. Only when red cells are exposed to extremely hypertonic concentrations (0.6 *M* glucose in 0.17 *M* NaCl-PO_4) is the permeability constant altered.

5. Slight previous shrinking of red cells does not alter the rate of penetration of glucose.

6. Chemical analysis of the plasma at various times provided measurement of the rate of penetration of glucose into the cells of slightly concentrated whole blood suspensions to which varying concentrations of glucose have been added. The volume changes these cells undergo as glucose penetrates were followed by hematocrit technique.

7. Absolute permeability constants have been calculated from the relative constants obtained by densimeter and chemical analytical techniques.

8. The results of both densimeter and chemical analytical experiments are interpreted as showing clearly that the rate of penetration of glucose (the amount of glucose which enters the cells per unit time) is determined by the external glucose concentration.

9. A discussion of factors that influence volume changes of erythrocytes and an analysis of some important aspects of the techniques employed in this as well as in previous investigations of this type is presented.

10. It is concluded that if human red cells are not washed or stored for long periods, but are kept in normal plasma and used fresh, and precaution is taken against introducing any experimental factor that may alter the ability of the cells to exhibit normal volume changes, glucose will penetrate in a manner predicted by the laws of simple diffusion.

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DIFFERENCES BETWEEN YOUNG AND MATURE RABBIT ERYTHROCYTES[1]

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FIVE FIGURES

It has been well known for a long time that the production of young red blood cells in mammals is enhanced when the animals become anemic (Wintrobe, '51). These new erythrocytes when stained with brilliant cresyl blue are recognized by a blue reticulum in the cytoplasm and have therefore been referred to as reticulocytes. All the young red cells do not possess a reticulum, but the reticulocytes are presumably the youngest red cells in the circulation.

In the past the physiological properties of young red blood cells have been inferred from investigations of anemic blood obtained from either humans with certain blood dyscraisas or laboratory animals with drug induced anemias. Erickson et al. ('37), Williams et al. ('41), Maizels ('36), and Farmer and Maizels ('39) used anemic human blood. The data derived from the cells of patients is certainly questionable when the information sought is for the normal young or mature erythrocyte. The use of the drug phenylhydrazine by Watson and Clark ('37), Dziemian ('42), and Gabrio and Finch ('54) to induce anemia in laboratory animals suffers from the same criticism. Under the foregoing conditions, in fact, Muirhead, Groves and Bryan ('54) and Rigdon, Micks and Breslin ('53) have experimentally verified the supposition that the circulating blood cells are directly altered by the treatment.

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Plum ('43) induced anemia in rabbits by repeated hemorrhage. With the precautions he has outlined, so as not to induce undue stress in the other organ systems of the organism, repeated bleedings of moderate quantities of blood seem to be the best approach to enhance normal young red cell production. This method, however, does not result in a high percentage of reticulocytes, and Plum used mixed cell populations for his studies.

The possibility of obtaining a more concentrated mass of young cells from a mixture of young and mature erythrocytes was suggested by the partial fractionation of reticulocytes that was achieved with low speed centrifugation or gravitational sedimentation by Key ('21), Watson and Clark ('37), Stephans ('40) and Keitel et al. ('55). As a result of these studies, Key ('21) deduced that the reticulocytes (from anemic rabbits) had a lower density than the mature red cells. Stephans ('40) using splenectomized dogs reported that the density of the young cells might be lower than that of the rest of the cells, but he maintained that the main reason that the young cells remained at the top of a column of sedimenting cells was the difference in the ability to form rouleaux or aggregates. He claimed that the young cells did not form rouleaux whereas the older cells did, and thus the density difference between the aggregated older cells and the non-aggregated young cells resulted in the fractionation that he observed.

Properties of the reticulocytes such as aerobic respiration (Wright, '30; Ramsey and Warren, '32; and Stephans, '40), electrophoretic mobility and osmotic resistance (Stephans, '40) differ both qualitatively and quantitatively from that of the mature cell. The work of Williams et al. ('41), Maizels ('36) and Farmer and Maizels ('39) with the pathological bloods presents data which indicate that young cells have more cations than mature cells per unit volume of packed cells. Their work showed that an excess of cations over mineral anions is present in the young cells, and they believe that the organic intracellular constituents make up the charge difference for the maintenance of intracellular electrical neutrality. Keitel et al. ('55) have reported similar data. In all these studies, however, the values were presented in such a way as to bear only indirect correspondence to the contents in equal number of cells.

Light microscope observations by Key ('21) and Doan and Ralph ('50) indicated that some young red cells stain with Janus green and Neutral red. The phase contrast microscope observations on unfixed cells by Ackerman and Bellios ('55) revealed the probable presence of mitochondria and cytoplasmic granules in the erythroid series in the bone marrow and in some circulating red blood cells.

Electron microscope studies on dried ghosts from mature red cells have been reported by Hillier and Hoffman ('53). These authors reviewed the existing physiological evidence and indicate that the ghost of the erythrocyte is comparable with the membrane of the intact cell. Mature red cells have been sectioned and observed with the electron microscope by Latta ('52), Bernhard ('52) and Kautz and DeMarsh ('53). No recognizable intracellular structures were observed and the reported values for cell membrane thickness varied from three to 20 times the thickness noted by Hillier and Hoffman ('53).

Electron micrographs of a replica of the ghost of a reticulocyte (Bessis, '50) and of dried reticulocyte ghosts (Braunsteiner and Bernhard, '50; Peters and Wigand, '50) show the remains of intracellular structures in the ghost but little detail can be discerned. No previous work dealing with sections of reticulocytes has been found in the literature.

The purpose of this work was to investigate the physiological and morphological properties of red blood cells as a function of their transformation *in vivo* from reticulocytes to mature cells.

MATERIAL AND METHODS

1. *Production of young cells*

Increased young blood cell production was induced in rabbits by repeated bleedings. Blood was withdrawn by heart puncture, usually in the morning before the rabbits were fed. When needed for the experiments to be described, it was collected into isotonic buffered saline containing dissolved sodium citrate (4 mg/ml blood). A blood volume equivalent to about 2% of the total body weight, i.e., 20–30% of the total blood volume, was withdrawn every third day. This amounted to 35–50 ml of blood, depending on the weight of the rabbit. Reticulocyte counts were made on every sample of blood and the first bleeding was used as the normal blood sample (control). After 9 days the reticulocyte count reached 15–20% and the blood sample was used for the harvesting of the young erythrocytes.

2. *Concentrating young cells*

The blood sample was centrifuged at 2,500 G for 10–20 minutes and the plasma and buffy coat removed. One milliliter aliquots of the resulting concentrated cell suspension (about 75–80% hematocrit) was then recentrifuged at 20,000 G for 20–30 minutes in the air turbine described by Parpart and Green ('51). The cells taken off of the top of the resulting packed cell column are referred to as ''top'' cells and similarly the cells taken from the bottom are the ''bottom'' cells. When larger quantities of ''top'' cells were desired the cells were centrifuged in a number of air turbine tubes. The ''top'' cells were pooled and then recentrifuged at the high speed.

3. *Analytical methods*

(a) Staining. A drop of 1% brilliant cresyl blue made up in isotonic saline was mixed with an equal volume of blood. A blood smear of this mixture was counterstained with Wright's stain and 1,000 red cells were counted.

(b) Cell counts. A 0.1 ml serological pipette graduated to the tip was used to withdraw measured packed cell samples from the 1 ml air turbine tubes. The packed cells were washed into 5 ml of isotonic NaCl-PO_4 (pH = 7.3). The cell counts were made with the standard red blood cell diluting (1:200) pipette and a hemocytometer. Four individual counts were made on each sample. One milliliter of this cell suspension in 10 ml of distilled water was used for hemoglobin determination.

(c) Hemoglobin determinations. A model DU Beckman spectrophotometer was employed and the optical density at 544 mμ of a red cell hemolysate was measured. Relative hemoglobin concentrations were determined in conjunction with the cell counts and the sodium and potassium analysis.

(d) Cell density and dry weight determinations. Bulk density measurements were made by weighing the 0.1 ml serological pipette with and without the packed cell samples. Knowing the weight of the 0.1 ml of packed cells, the density of the cells was calculated.

The cells in the pipette were washed with distilled water into tared glass vessels. The cell residue was frozen and then dried overnight *in vacuo* over $CaSO_4$. The process was repeated until a constant dry weight was obtained.

(e) Cell volume determinations. The number of cells per volume of packed cells as determined in 3 (b) is a measure of the average red cell volume. With the assumption that the amount of hemoglobin per red cell is the same for all the circulating red cells (see Results) the ratio of the optical density at 544 mμ was used to determine the relative volume of the cells. The hemoglobin determinations are more accurate and easier to perform.

(f) Water content. The combination of the dry weight, the wet weight [see 3 (d)] and the cell count [see 3 (b)] made it possible to calculate the water content per volume of cells or per average cell.

(g) Sodium and potassium analysis. One-tenth milliliter of packed cells was quantitatively transferred to 15 ml of

distilled water, thereby hemolyzing the cells. One milliliter of 25% Trichloroacetic acid (TCA) was added to 5 ml of the hemolysate. The precipitate was centrifuged and the clear supernatant solution was used for the sodium analysis. The potassium determinations were made using a half dilution of the clear supernatant. A TCA blank was always made up in the same dilutions as the unknowns.

A Beckman spectrophotometer with flame attachment was employed.

One milliliter of hemolysate was added to 10 ml of distilled water and used for hemoglobin determinations [see 3 (c)] (Parpart and Green, '51).

(h) Osmotic resistance measurements. Twenty cubic millimeters of the cell suspension in 3 (b) were added to a series of hypotonic saline solutions in accordance with the procedures and precautions outlined by Parpart et al. ('47). The hemoglobin concentration due to the great dilution in this type of determination was measured by the optical density at 413 mμ rather than at 544 mμ.

4. Electron microscope methods

Red cells which had been fractionated into "top" cells and "bottom" cells were washed twice with isotonic buffered $NaCl\text{-}PO_4$ ($pH = 7.4$). Clean ghosts of portions of these two fractions were prepared by repeated osmotic hemolysis according to the method outlined by Hillier and Hoffman ('53).

Fixation as described by Palade ('52) was carried out by placing the cells in 2% Osmium tetroxide dissolved in 1% (isotonic) $NaCl\text{-}PO_4$ ($pH = 7.4$) for ca. two hours. The cells were washed with isotonic saline, distilled water and then dehydrated by means of a graded alcohol series. The cells were embedded in a 3:1 catalyzed mixture of butyl and ethyl methacrylate in dry gelatine capsules after having been washed with an alcohol-methacrylate mixture and then several times with the catalyzed monomer mixture. Polymerization was effected by maintaining the gelatine capsules at 47°C. overnight.

Sections were cut with glass knives using the microtome developed by Hillier and Chapman (Chapman, '53). The thickness of the sections were estimated from the color reflecting from the surfaces of the sections as described by Porter and Blum ('53).

An RCA type EMU electron microscope with a wide field objective and a 50 μ copper aperture was used. The micrographs were taken at an electron optical magnification of 9,500 × and then photographically enlarged.

RESULTS

Reticulocytes have been reported to come to the centripetal ("top") end of a centrifuged column of cells. The extent

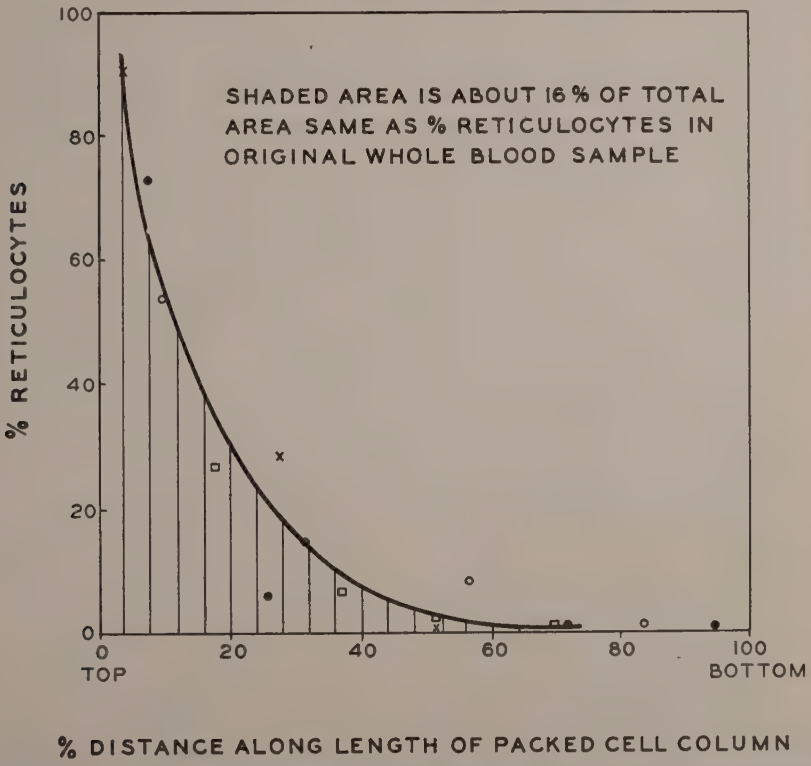

Fig. 1 The distribution of the reticulocytes along the length of the packed cell column.

of the separation between the reticulocytes and the rest of the cell population was determined by sampling a small amount of cells at different levels along the packed cell column. Figure 1 is the result of 4 independent determinations indicating

TABLE 1

	NO. OF DETERMINATIONS	AVERAGE VALUES	
		Top level (young cells)	Bottom level (older cells)
1. Optical density of hemoglobin solution at 544 mμ	4	0.121 (0.118–0.138)	0.169 (0.167–0.173)
2. Cell count (10^9 cells/ml cells)	4	10.6 (10.4–10.9)	15.2 (14.3–15.4)
3. Bulk density of cells (gm/ml cells)	4	1.105 (1.103–1.107)	1.122 (1.121–1.123)
4. Dry weight (gm/ml cells)	4	0.435 (0.444–0.427)	0.359 (0.357–0.363)
Calculated results			
5. Per cent dry weight		32.5	39.0
6. Per cent water		67.5	61.0
7. $\frac{\text{Weight of water}}{\text{ml cells}}$ (gm/ml cells)		0.770	0.763
8. Volume of average red blood cells (μ^3)		96.1	65.0
9. Water volume/cell (μ^3/cell)		64.9	39.7
10. Dry weight of average red blood cell (10^{-11} gm/cell)		3.45	2.89
11. Density of cell solids (gm/cm^3 solid)		1.422	1.205
12. Relative cell volume		1.48	1.00

that cell samples containing 80–100% reticulocytes can be obtained from the top of a centrifuged packed red cell column (with the buffy coat previously removed) from blood which has an overall reticulocyte count of around 16%.

Measurement of the bulk density (table 1) shows that the young cells have a lower density. The spread in the density values for the young cells is from 1.103 to 1.107 and for the older, mature cells from 1.121 to 1.123 gm/ml packed cells. This does not rule out the possibility that rouleaux formation of the older cells does occur and enhance the separation (Stephans, '39).

The osmotic resistance measurements of the young and older cells are shown in figure 2, and as can be seen the younger cells are more resistant to osmotic hemolysis than are the older cells. The increased resistance can be explained, and would be consistent with the observation that the young cells have a greater cell volume, provided the younger cells also have a ratio of thickness to diameter smaller than the mature cell. Light microscope examination of both cell types confirms these assumptions and thus support this hypothesis. Under these conditions it would take a more dilute salinity for the larger reticulocyte to reach a prehemolytic spherical shape than would be needed for the smaller mature cell. Thus the greater osmotic resistance of the younger cells.

The average size of the red cell can be calculated knowing the number of cells in a given volume of packed cells. The data presented in table 1 showing the number of cells in 1 ml of packed cells were used for such a calculation and indicate that the reticulocytes have a cell volume approximately 1.5 × that of the mature erythrocytes.

The dry weight of the reticulocytes (table 1) is 0.431 gm per milliliter of cells and for the mature cells 0.359 gm/ml cells. The calculated values for the per cent water, the amount of water and the dry weight per average red blood cell were obtained by using this measurement in conjunction with the wet weight (bulk density) and the cell counts.

The values for the hemoglobin content were used in the cation calculations so that the data would present the amount of cations in an equal number of cells. Obviously this necessitates the assumption that the quantity of hemoglobin per cell regardless of cell age remains constant. Such an *a priori* assumption cannot be made; however, to test this, cell counts and hemoglobin content were measured and the values are reported in table 1 in the first two lines. In the 4 determinations reported for the cell count versus hemoglobin content, the average per cent difference is 2.9%. The spread of the per cent difference for the 4 determinations is from 1.8% to

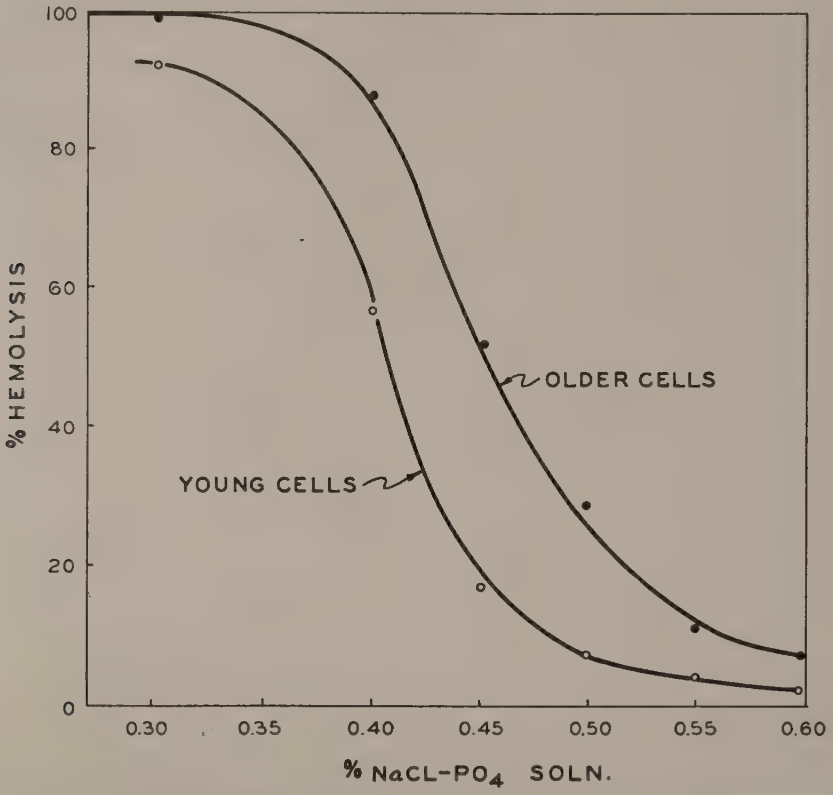

Fig. 2 Osmotic resistance (fragility) of the "top" (young) cells and "bottom" (mature) cells. The "top" cells are more resistant to osmotic hemolysis than are the mature cells.

4.7%. These differences are about the amount of experimental error involved in this type of a determination, and therefore it is concluded that the hemoglobin or rather heme-chromogen content per cell of the circulating red cells is the same and does not change as the reticulocyte matures. The use of hemoglobin content as an indication of cell number seems justified in the calculations for the cation determinations; also as previously indicated hemoglobin can be determined more accurately than cell counts.

It is important to recognize with what frame of reference these results are reported. Such measurements as per cent water content, or cation content in the blood cell samples are listed, at times, without relevance to the entity of greatest interest, i.e., the average single blood cell (Keitel et al., '55, for human red blood cells). Table 2 and the lower portion of table 1 show a series of calculated values which were obtained from the single set of data presented in the top of table 1 and figure 3. Comparison of the water content of reticulocytes and mature erythrocytes (table 1) give diverse results depending on the indices employed. They appear to be about the same when compared as weight per volume of packed cells. They are slightly different when judged from per cent water (volume of water per volume of packed cells). They are considerably different when compared as to volume of water per volume of average single cell. These variations are also true for the dry weight calculations. The data as related to the average single cell, or to an equal number of cells, are the most significant.

The results of the sodium and potassium analysis along a packed cell column of anemic blood are presented in figure 3 and it is to be noted that these results reveal the amount, not the concentration, of cations per unit number of cells. The concentration of cations are found to be about the same, regardless of the age of the cells (table 2, line 2).

Each cation value is the average of 5 determinations. The standard deviation for K is ± 4.78 meq and for Na is ± 2.71 meq and corresponds to about a 3% error for K determina-

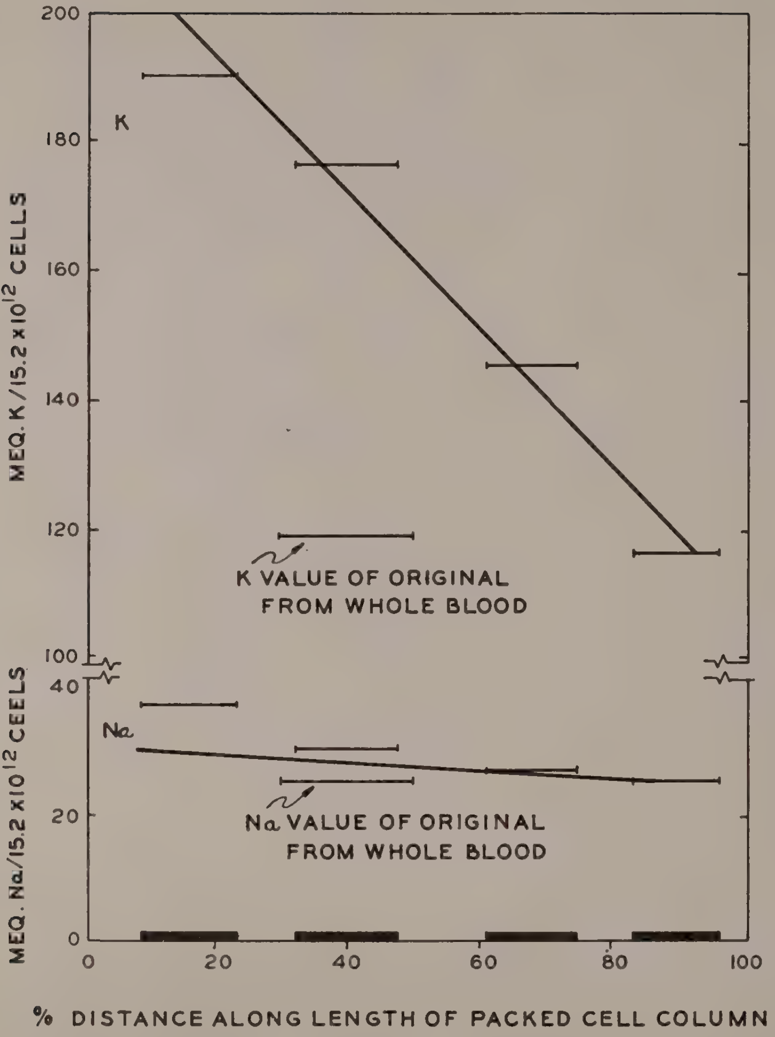

Fig. 3 The sodium and potassium content of rabbit red cells along the length of a centrifuged packed cell column of anemic blood. The values for the mature red cells taken from the first bleeding are also shown. The dark areas along the abscissa indicate the quantity of cells removed for a determination.

One liter of packed mature cells has 15.2×10^{12} cells. This is not the case for the young cells. (See table 1.) 15.2×10^{12} cells is used as the reference for the cation values in this figure.

The standard deviation for the K values is ± 4.78 meq K and for the Na values is ± 2.71 meq Na.

TABLE 2

MILLI-EQUIVALENTS PER	FROM ANEMIC BLOOD								FROM WHOLE BLOOD			
	Reticulocytes Top layers				Mature cells Bottom layers				Original bleeding			
	K	Na	K + Na	K/Na	K	Na	K + Na	K/Na	K	Na	K + Na	K/Na
1. Liter cells	134.2	24.8	159.0	5.4	118.9	26.9	145.8	4.4	118	26.8	144.8	4.4
2. Liter cell water	198.8	36.7	235.5	5.4	193.3	44.1	237.4	4.4	193	43.9	236.9	4.4
3. Kilogram dry weight	308.5	57.0	345.5	5.4	330.5	75.0	405.5	4.4	328.5	74.7	403.2	4.4
4. 15.2×10^{12} cells	191.0	35.1	226.1	5.4	121.6	27.6	149.2	4.4	118	26.8	144.8	4.4
5. Average single cell	12.56	2.3	14.86	5.4	8.0	1.81	9.81	4.4	7.76	1.76	9.52	4.4

tions and about 10% error for Na determinations. The data presented in this way refer to the amount of potassium within the cell as a function of the density of the cells. As is mentioned further along, the cell size variations, and the distribution of reticulocytes as well as the cation content, tends to lend support to the hypothesis that the density difference in these erythrocytes is proportional to the cell age. The lower part of the same figure gives the amount of Na per number of cells at different levels. The ratio of K/Na (table 2) is 5.4 for the reticulocytes and 4.4 for mature (bottom) cells.

The readings from the flame photometer are in per cent of a standard cation solution with which the instrument is calibrated. The concentration of cations is read from a calibration curve. The following calculations were used to arrive at the values presented in table 2:

$$\frac{\begin{array}{c}\text{cation concentration}\\ \text{of unknown}\end{array} \quad \begin{array}{c}\text{dilution factor}\\ \text{of unknown}\end{array}}{\text{(equivalent weight of cation)}} = \frac{\text{meq of cation}}{\text{liter of cells}} \qquad (1)$$

The "dilution factor" is the number of dilutions that were necessary in order to dilute the packed cells to the point where the sample was used in the flame photometer. For sodium it is 181.2 × and for potassium 362.4 ×. Other calculations were made as follows for each cation:

$$\frac{\text{(meq of cation/liter of cells)}}{\text{(\% water) (100)}} = \frac{\text{meq of cation}}{\text{liter of cell water}} \qquad (2)$$

$$\frac{\text{(meq of cation/liter of cells) (1,000)}}{\text{(grams of dry wt./liter of cells)}} = \frac{\text{meq of cation}}{\text{kilogram dry wt.}} \qquad (3)$$

A liter of packed mature cells was found to contain 15.2×10^{12} cells, whereas there are fewer cells in a liter of young cells, viz., 10.4×10^{12}. Calculation of the amount of cations in the same number of cells (knowing that the hemoglobin [Hb] content will be in direct proportion to cell number) were made as follows:

$$\frac{\text{(meq of cation) (Hb content of mature cells)}}{\text{(liter of cells) (Hb content of young cells)}} = \frac{\text{meq of cation}}{\begin{array}{c}\text{No. young cells equiva-}\\ \text{lent to No. mature cells}\\ \text{in a liter of packed cells}\\ (15.2 \times 10^{12})\end{array}} \qquad (4)$$

$$\frac{\text{(meq cation/No. of cells)}}{\text{(No. of cells)}} = \frac{\text{meq of cation}}{\text{average single cell}} \qquad (5)$$

The values of interest in the above respect are in lines 4 and 5 of table 2. A more realistic comparison of the cation contents of young and mature erythrocytes can be derived from these lines. Lines 1 and 3 are included since there are some values in the literature which uses such connotations (Keitel et al., '55). Line 2, third column in each case in table 2 is the total concentration of cations in the water of the cells and, as can be seen, there is essentially no osmotic inequality between young and mature cells.

The upper part of figure 3 presents the K content per unit number of cells at the different levels along the packed column of cells. The amount of K per liter of mature cells from the original bleeding is also shown. The horizontal lines show the amount of material taken for a given determination.

The hemoglobin readings in optical density at 544 mμ as used above in equation (4) are for the sample from 8–22% along the cell column (the darkened areas on the abscissa) equal to 0.152, from 33–47% = 0.167, from 61–73% = 0.179, and from 84–97% = 0.212. The reading for the original control was 0.209.

Since serum contains a large amount of sodium but very little potassium, any contamination of the top layers by serum would tend to dilute the potassium to yield lower potassium values and increase the sodium with the resulting higher values for sodium. To check this, cells from the various levels were withdrawn and recentrifuged at high speeds in the air turbine. The values obtained after this treatment indicated that there was a small amount of serum contamination only in the top layers and the lines drawn in on figure 3 indicate the direction of this correction for the sodium and potassium values.

Electron microscope results

Plates 1 A, 1 B and 2 illustrate sections of reticulocytes. These cells contain structures which have not been observed in the mature cells.

A single section of a red cell reveals only about 3% of the whole cell volume. Therefore, the absence in a given micrograph of internal structures is certainly no proof that such structures were not in the whole cell. This assumes increased importance when it is remembered that the total number of electron micrographs in comparison to the total number of cells contained in a single blood sample was quite small. Approximately a thousand micrographs were taken, two-thirds of which were of sectioned reticulocytes. In general, however, the evidence to be presented confirms the results obtained in the past with light microscopy and physiological studies and indicates the presence, in addition to the cell membrane, of mitochondria-like structures, granules and/or vacuoles. The cell membrane measures 60–70 Å thick. This value is the same as that obtained for the mature cell.

A section of an osmium fixed ghost of a reticulocyte prepared with hypotonic solutions (plate 1 C) shows that the cell membrane observed is the same thickness as in the intact cell. The ghost retained a number of internal structures. These structures bear little resemblance to those in unhemolyzed red cells. That there are structures remaining within the reticulocyte ghost has been ascertained with the light microscope. Interpretations of chemical studies on the composition of the ghosts of young erythrocytes should take into consideration the existence of this internal material.

The structures containing laminated membranes in plates 1 A, 1 B, and 2 are smaller but otherwise correspond well morphologically with the mitochondria found in other cells (Sjöstrand, '53; Rhodin, '54; Palade, '52a). The fact that young red blood cells consume oxygen (Ramsey and Warren, '30) and are able to concentrate Janus green B (Ackerman and Bellios, '55) lends confirmatory evidence that these structures correspond physiologically to the mitochondria in other cells. These mitochondria-like structures are bordered by and made up of double layer membranes. The membranes are composed of two denser outside layers with a less dense area between them. The overall thickness of the membrane is about 200 Å. The denser outer layers are about 70 Å thick with the less dense area not as uniform in thickness: 35 to 75 Å. The composition of these layers have yet to be worked out and can only be suspected from such information as the electron density (Bahr, '54) and the chemical composition of the cell membrane found for mature red cells (Parpart and Ballentine, '52).

Several granules containing osmophilic material adjacent to several apparently empty vacuoles can also be seen in plate 1 A. These vacuoles could also possibly be either a transient stage or a tangential cut of a granule. A few of the granules and/or vacuoles observed are large enough to be discerned with the light microscope. Many, however, are smaller than the resolving power of the light microscope and would only be revealed with the electron microscope. These micrographs, therefore, are the only evidence for their possible existence in the untreated cell.

The plane of section through a single cell is random and the resulting sizes and shapes vary considerably in the micrographs. It is clear that no critical inference as to the actual size of the whole cell can be reconstructed from a single micrograph. However, mature red cells are more uniform in shape and the sections of these cells indicate that they could be derived from a biconcave disc shape. In the case of the reticulocytes, however, the shape of the cells is not as obviously a biconcave disc. Observations of reticulocytes with the light microscope confirm the idea that the reticulocytes maintain a more or less flattened disc shape but are also quite poikelocytotic. The preparation of the young cells for sectioning seems to fix the cells in variable shapes. Plate 2 is such an example. It is hard to visualize this as a section of a disc shaped structure. The shape observed could have been induced by the preparative treatment or actually have been the shape of the cell when it was fixed. The latter view would be favored if the fixation of the cells occurred more rapidly than possible osmotic changes. The observation that mature cells when fixed with osmium in *distilled water* maintain their biconcave shape, supports the idea that fixation is more rapid than osmotic shape changes.

Along the surface of reticulocytes as seen in plates 1 A, 1 B, and 2 small indentations are sometimes observed which have not appeared in sections of the mature cells. The number of these indentations per thin section varied from one to 4. They have a dense membrane about 150 Å thick at the base of the indentation. The juxtaposition of these structures to such recognizable intact structures as the mitochondria could be indicative that their existence is not a product of the procedures employed. Also observed, but in fewer micrographs, are small "rings" about 700 Å in diameter made up of a membrane about 150 Å thick. These structures appear to reside around the periphery of the cell. They could possibly be the result of a section in another plane of a surface indentation.

The micrographs of sections of mature erythrocytes reveal a cell membrane of the same thickness as in the reticulocyte, i.e., 60–70 Å thick. No readily distinguishable internal structures have been observed as was seen in the young cells. The cytoplasm, mostly hemoglobin, appears to be undifferentiated with respect to structure.

Micrographs of sections of mature red cell ghosts again reveal the thickness of the cell membrane to be 60–70 Å thick with no observable internal remnants.

In the micrographs of sections of cells (as in plates 1 and 2) the cell membrane does not appear continuous along the periphery of the sectioned red cell. Hillier and Hoffman ('53) have shown, however, that the cell membrane completely surrounds the cell and has a uniform thickness. As can be seen in plate 1 C the cell membrane seems more diffuse in some areas and more compact in others. A tangential cut so that the membrane was lying flat in the section would explain the appearance of these diffuse areas. These places would be obscured in sections of whole cells due to the very high electron density of the cytoplasm.

It is because of this very high electron density of the cytoplasm of osmium fixed cells that extremely thin sections are essential in order to discern cellular details in erythrocytes. The necessity for extremely thin sections when studying red cells is most clearly illustrated in micrographs of tissue sections in which red cells appear (Low, '54; Van Breeman and Clemente, '55).

GENERAL DISCUSSION

The dry weight of the reticulocytes is 0.431 gm per milliliter of cells and for the mature cells 0.359 gm/ml cells. This is comparable to the Boyle-Van't Hoff "b" value for the osmotic properties of the cell (Parpart and Shull, '35). The average reticulocyte has about 0.56×10^{-11} gm more dry weight per cell than the older mature cell. This loss as the cell matures cannot be attributed to hemoglobin diminution per cell since it was shown that the hemoglobin content remains constant for all the circulating red cells. The loss in dry weight cannot be accounted for by the decreased amount of salt in the older cells since the difference in the cumulative sodium, potassium and chloride weight is only 5% of the total difference in dry weights. Watson and Clark ('37) have demonstrated that reticulocytes contain protoporphyrin. This claim is also substantiated by fluorescence microscopy (Popper and Szanto, '50) with which the reticulocytes have been shown to fluoresce. The reticulum which is observed when the young cells are stained with brilliant cresyl blue is not apparent in unstained preparations and is thought to be a precipitation of the protoporphyrin by the dye. In any event, the protoporphyrin disappears as the cell matures and may contribute to the weight difference observed. Since protoporphyrin is most likely converted into hemoglobin it does not appear that the weight difference would be due to the loss of protoporphyrin as such. Dustin ('44) and Brachet ('50) report the presence of a much larger quantity of nucleoprotein in young red cells than in mature cells. This may also contribute to the dry weight differences. Cytoplasmic structures which have been demonstrated in the young cells disappear as the cell develops. These structures are undoubtedly responsible for a considerable portion of the observed difference in dry weight.

Two possible ways of interpreting the intermediate values for cell size and cation content found between the top and bottom portion of the cell column are (1) that the result of varying combinations of only two existing cell types, young and mature cells, yield a gradient only of the degree of mixture along the cell column, or (2) that the cells of different age or composition are resolved into strata along the cell column. An analysis of the data shows that the reticulocyte distribution does not parallel the distribution of cell size or of cation content in the same column of cells (figs. 1 and 3, as well as page 229). For example, 40% from the "top" of the cell column, the reticulocyte count showed 90% nonreticulated cells; however, the average cell volume (i.e., hemoglobin content) is roughly 63% of the "top" cells and the cation content about 65% of the "top" cells. This evidence suggests that the intermediate values for size or cation content is not the result of a mixture of only two cell types but is the result of cells which are individually intermediate in developmental stages between the very young reticulocytes and the older mature erythrocytes.

The results obtained with the electron microscope presents additional evidence, by an independent physical method, of the existence of the cell membrane. The thickness of the membrane is the same in the whole cell as in the sectioned ghost, and corroborates the thickness found by Hillier and Hoffman ('53).

The 10 to 15% shrinkage involved in the preparative procedure for sections does not alter the orders of magnitude of membrane thickness dealt with here. Given no differential shrinkage within the preparation, a 60 Å membrane in the micrograph would have been 70 Å *in vivo,* i.e., not significantly altered. The values for the red cell membrane arrived at by Kautz and DeMarsh ('54) of 200 to 300 Å and by Bernhard

('52) of 160 to 320 Å using sectioned material are about twice or three times the values obtained in this study. Latta ('52) arrived at a membrane thickness from 200 to 1,000 Å using sections from which the embedding was removed and subsequently metal shadowed. Citing physiological evidence, Ponder ('49) reported the red cell membrane thickness to be 125 Å. Parpart and Ballentine ('52) calculated, from their own data, a cell membrane thickness of 52 Å. The differences in these values are negligible when compared to the proposed 5,000 Å membrane by Mitcheson ('50) derived from polarized light microscopy.

The membrane thickness of the mature cell is the same as that for the young cell. This equivalent was also claimed by Ruhenstroth-Bauer and Schmidt ('54) using an interferometric microscope method on saline suspended ghosts; however, they report a membrane thickness of 150 to 240 Å.

Sub-light-microscopic morphology within the young red blood cell has been partly revealed. The Janus green uptake and to a lesser extent the higher rate of oxygen consumption of the young cells could possibly have led to the prediction of the mitochondria found. Any attempt to delineate the functions of the other intracellular structures, however, would be speculative without further study. The final stages of the hemoglobin synthesis from free protoporphyrin may take place in the granules (Brachet, '50), especially those granules with the observable osmophilic contents (Bahr, '54). Neutral red which is concentrated in granules or vacuoles of the young red cell (Baker, '50) presumably would reside in structures comparable to these observed with the electron microscope.

The mechanism of red cell development in the circulation appears to be a loss of many of the highly specialized living structures which in other cells seem to be used for the maintenance of the life functions. The mechanism of the loss from the cell of large protein molecules such as the RNA-protein and the oxidative respiratory enzymes during red cell maturation must be left to speculation. The structures and/or proteins could possibly be degraded enzymatically and the products diffused out through the cell membrane. The granules, vacuoles and mitochondria could also be expelled from the cell by breaking through the cell membrane. Little evidence can be cited in support of either of these possibilities (Wintrobe, '51).

As is seen from the K/Na ratio in the reticulocytes and in the mature cells, proportionately more K than Na is lost as the cells age *in vivo*. The main loss of cations is due to the K loss since the total amount of Na lost is quite small. The decrease in cell water during cell maturation could easily be conceived as the osmotic adjustment of the cells to the loss of K. Thus as the cell ages there is a decrease in the membrane surface area and cell volume. Further work will be required to clarify the process of *in vivo* erythrocyte aging.

I am indebted to Professor Arthur K. Parpart and to Dr. Joseph F. Hoffman for their continued stimulation and aid throughout the course of this work. I would also like to record my thanks to Dr. George B. Chapman and to the RCA Laboratory Division, David Sarnoff Research Center at Princeton, New Jersey, for their kindness in placing at my disposal certain of their facilities.

SUMMARY

1. The young red cells whose production had been enhanced by repeated hemorrhage are less dense than mature erythrocytes and can be concentrated in the top of a centrifuged column of cells.

2. The young circulating red cells are ca. 1.5 × as large as the mature cells but contain about the same amount of hemoglobin per cell.

3. The cation content per cell is considerably higher in the young cells than in the mature erythrocytes; however the cation concentration is the same in both. The K/Na ratio is 5.4 in the reticulocytes and 4.4 in the mature cells, indi-

cating that as the young cells mature proportionately more K than Na is lost from the cells.

4. The young cells are more resistant to hypotonic hemolysis than are the mature cells. This has been ascribed to the size and shape differences between the cell types.

5. Ultrathin sections of reticulocytes and mature erythrocytes were studied by means of the electron microscope.

6. Sections of reticulocytes revealed a cell membrane 60–70 Å thick.

7. Sections of mature red cells revealed a cell membrane similar to that of the reticulocytes. No internal structures were observed.

8. Intracellular structures were observed in the reticulocytes. Laminated mitochondria-like structures made up of double layered membranes are described. The two outer dense layers are about 70 Å thick and the less dense middle area are of variable thickness: 35–75 Å. The structure is bordered by a similar layered membrane. Granules 0.3 μ in diameter with osmophilic contents and vacuoles without much osmophilic material ranging from 0.3 μ to 0.03 μ were noted.

9. The relationship between the physiological and morphological data and the process of erythrocyte development in the peripheral circulation *in vivo* is discussed.

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PLATE 1

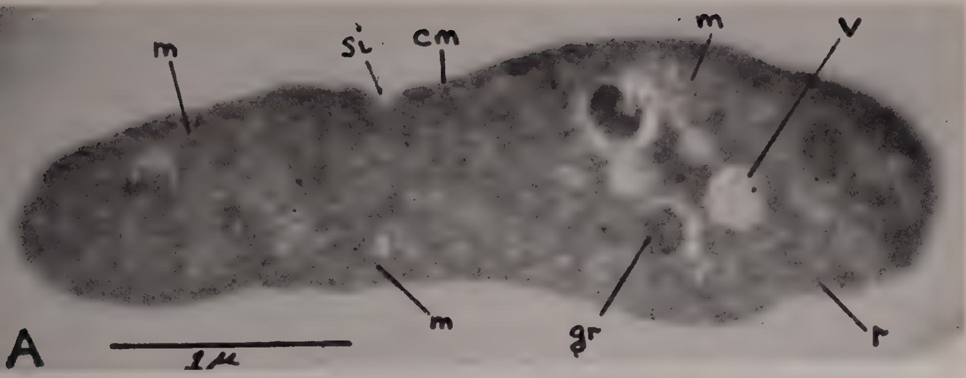
EXPLANATION OF FIGURES

A (507b) This section of a rabbit reticulocyte shows the 60–70 Å cell membrane (cm), a surface indentation (si), several small ring structures (r), vacuoles (v), granules (gr) with osmophilic contents, and mitochondria-like (m) structures containing double layered membranes.

The thickness of the membrane at the base of the surface indentation is about 150 Å thick. The small ring structures are about 700 Å in diameter and the thickness of the ring membrane is about 150 Å.

B This is a 2 × enlargement of a portion of the above micrograph. The double layered membrane (dlm) in the mitochondria are about 200 Å thick. Each of the layers is about 70 Å thick with the less dense area between them varying from 35–70 Å in thickness.

C (482c) This is a section of an osmotically prepared young cell (reticulocyte) ghost. The cell membrane (cm) is about 60–70 Å thick. The diffuse appearance of the membrane in certain areas (dcm) could be the result of the membrane lying somewhat flattened in the section. The internal structures (s) are most likely altered in appearance from that of the unhemolyzed reticulocyte.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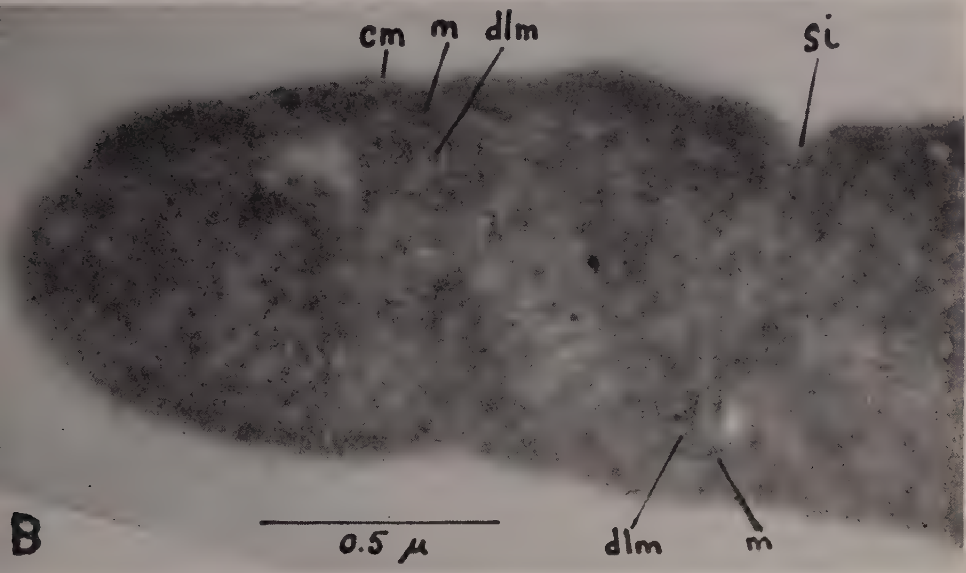
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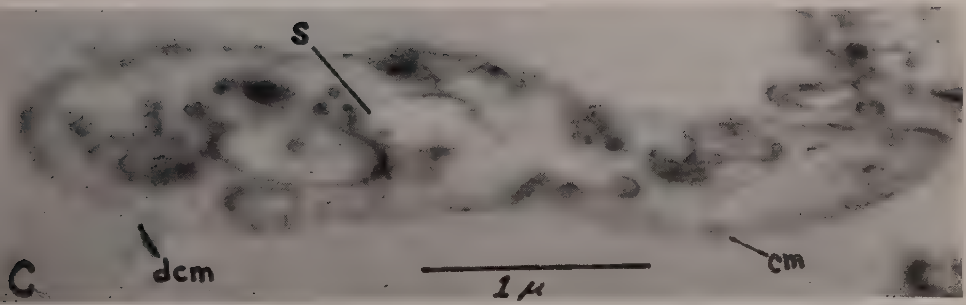
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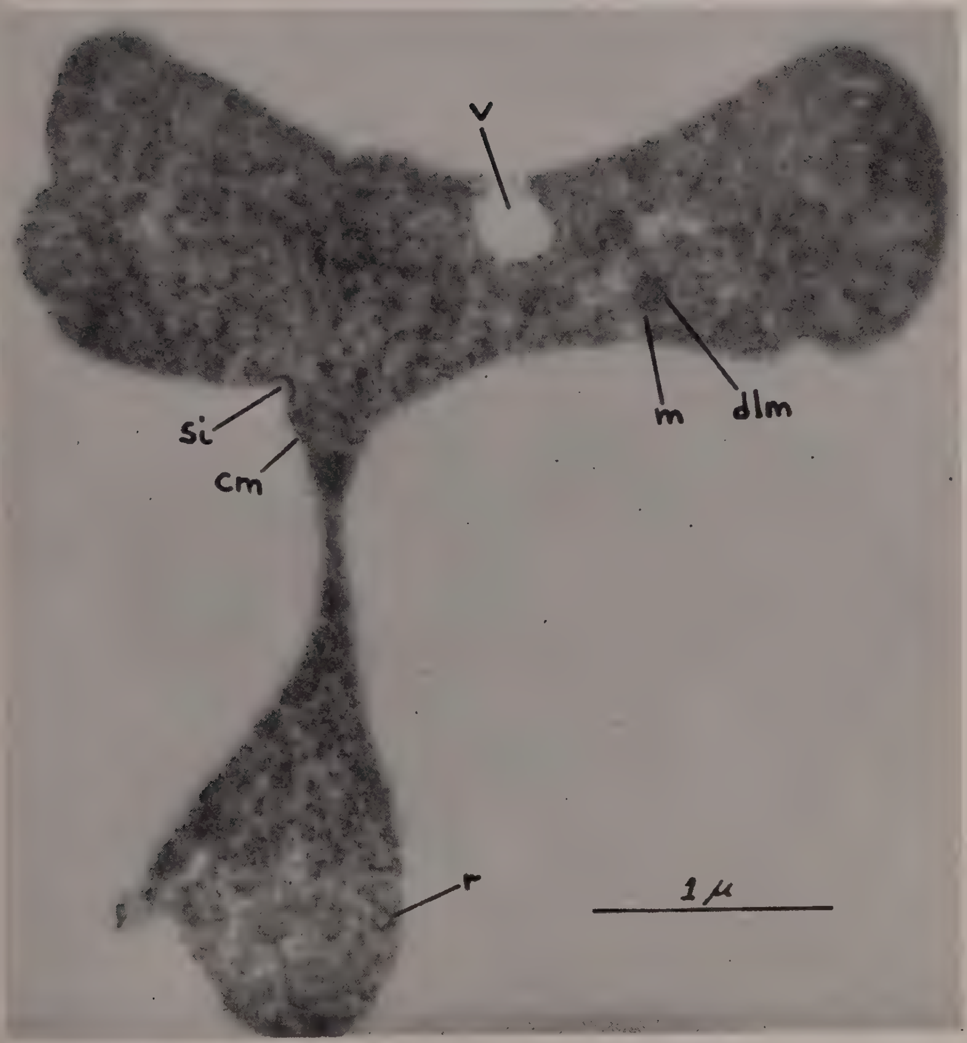
PLATE 2

EXPLANATION OF FIGURE

(476b) This is a section of a reticulocyte. It is difficult to visualize this overall shape as the result of a section through a biconcave disc. The cell membrane (cm) is not observed except in one or two places. Surface indentation (si), small ring structure (r), a vacuole (v), and mitochondria-like structures (m) can be seen. The double layered membranes (dlm) can be clearly seen in some of the mitochondria. The vacuole could be construed as having some association with the exterior of the cell. This could be a result of the manipulations involved in preparing the cell for sectioning.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NEW HIGH DENSITY PARTICLES IN CERTAIN NORMAL AND ABNORMAL ERYTHROCYTES[1]

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THREE FIGURES

INTRODUCTION

The techniques formerly devised to study the surface ultrastructure of the normal human ghost (Hillier and Hoffman, '53) have been employed to survey certain human erythrocytic anemias and a number of species of mammals. During the course of this work it was repeatedly observed that particles of high electron density were present in ghosts of certain species of mammals and some human hemolytic anemias. These have not been reported previously. Therefore, the purpose of this article is to describe the size, occurrence and distribution of these high density particles (HDP) as they appeared in various samples of blood of different origin.

MATERIALS AND METHODS

A small sample of blood was collected from humans, rabbit, rat, and groundhog in the manner described by Hillier and Hoffman ('53). Mouse blood was obtained from the snipped tail, bat blood from a clipped wing tip, camel[2] blood from a jugular vein, beef, sheep, and pig blood from the abattoir.

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[1] This work was in part supported by the Whitehall Foundation.

[2] Provided through the kindness of Dr. Charles P. Gandal of the New York Zoological Society.

Since in the case of camel, beef, pig, sheep blood and a number of the hemolytic anemias, the blood samples were collected some distance from the laboratory an effort was made to minimize the length of time between withdrawal of the samples to the terminal drying of the ghosts. In no instance was this interval greater than 6 hours. When possible the experiment was designed such that 4 experimental bloods and two controls were prepared at any one time. The control samples were taken from the same person (JFH); the first collection of blood was made concomitant with the bleeding of patients or animals; the second just prior to the preparative procedure. No differences could be discerned between these two control samples.

Two modifications were used in the application of the method of successive hemolysis (cf. Hillier and Hoffman, '53): (1) all centrifuging was carried out with 50 cm^3 glass-stoppered Pyrex tubes. The volume of fluid was kept the same, i.e., 20 cm^3. Recovery on the collodion membrane was approximately 90% of ghosts expected; (2) since the osmotic resistance of a number of the pathological bloods is known to be greater than normal blood the initial hemolyzing concentration of sodium chloride was adjusted such that 100% hemolysis was achieved with the first hemolytic wash. This could also be achieved by increasing the number of successive hemolyses. For the most osmotically resistant cells (Thalassemia major) no more than 6 were found necessary to obtain "clean" ghosts.

The high density particles to be described are best observed in the air-dried untreated specimen. However, staining and fixing with phosphotungstic acid or shadowing with chromium or both did not influence the general results reported below. All preparations were examined with an RCA Model EMU electron microscope fitted with a high resolution double objective.

RESULTS

The ghosts isolated from patients with Thalassemia major contained HDP in the greatest abundance yet observed. Figures 1 and 2 are representative ghosts from two such patients. Both micrographs show HDP associated with the ghost and no HDP on the supporting collodion. HDP were occasionally found outside the boundaries of the ghost but invariably were near a ripped or cracked edge. The most striking difference between the two micrographs is the range of size of the HDP. In figure 1 they appear of uniform dimension, being about 100 A in diameter. In figure 2 the range is from 40 to 100 A. The electron density is seen to vary according to the size. The appearance of the HDP per se as illustrated by these two pictures is characteristic of all HDP occurring in any of the bloods examined.

Table 1 lists the various types of blood studied and indicates whether or not HDP were found. Also tabulated are the number of individuals examined. When, in a few instances, a patient's blood was obtained a second time, no change from the former result was apparent.

HDP are generally not observed in normal (meaning no known blood dyscrasia) human ghosts. In one of the 6 normal individuals used in this study HDP were detected. This was the control blood (JFH) which has received the most intensive study. In approximately 3,000 micrographs of this blood, less than 10 ghosts showed HDP, and in these instances the population density was only a small fraction of that in figures 1 or 2. Thus, the occurrence is considered rare and is listed in the "not found in" column of table 1. On the other hand, this result with the control blood permits the interpretation that the presence or absence of HDP is not an all-or-none phenomenon but rather one of degree.

The proportion of ghosts showing HDP is quite variable for any sample of blood. In addition the number of HDP per ghost varies from ghost to ghost and depends upon the particular sample of blood. These relations in the case of ghosts from humans exhibiting one or another type of anemia seem to depend upon at least the following factors: (1) the severity of the anemia; (2) the heterozygosity or homozygosity of the subject; (3) therapeutic history, e.g., transfu-

TABLE 1

The occurrence of high density particles in isolated ghosts

HDP NOT FOUND IN	NUMBER OF CASES	HDP FOUND IN	NUMBER OF CASES	APPROXIMATE PER CENT OF GHOSTS WITH HDP	APPROXIMATE NO. OF HDP PER GHOST
Normal human	6	Thalassemia major	8	50	10^4–10^5
		Thalassemia minor	7	30–40	10^3–10^4
Iron deficiency anemias					
Macrocytic	2	Sickle cell carriers (SA)	2	70–80	10^2–10^3
Normocytic	1	Sickle cell disease (SS, SC)	6	80	10^2–10^3
Microcytic	4	Erythroblastosis fetalis	1	70–80	10^3
Lead Poisoning	1	Ovalocytosis	1	15	10^2–10^3
Polycythemia vera	2	Congenital hemolytic icterus	4	90–100	10^2–10^4
Lymphocytic leukemia	2	Pernicious anemia	3	50	10^2–10^3
Groundhog	2	Bat	1	80	10^3
Rabbit	2	Beef	2	100	10^3
Rat	1	Camel	1	100	10^4
		Mouse	1	20–30	10^2
		Pig	1	100	10^3
		Sheep	1	30–40	10^2–10^3

sion; (4) any selection or loss which preparative procedure may have imposed.

The relative proportion of ghosts containing HDP in the various samples is indicated in table 1. The range noted represents the per cent of the total ghosts photographed (usually between 20 and 50) that possessed HDP. This must be considered as semiquantitative since the method of sampling has not been evaluated. It is not possible to see the HDP without photographing the ghosts nor can one anticipate the presence of HDP in a ghost by its gross appearance on the fluorescent screen in the electron microscope.

All of the patients with Thalassemia major had been routinely receiving whole blood transfusions. Thus a considerable number of "normal" cells were present in the circulation. Since no attempt was made to separate the patient's blood from the donor blood, the final preparation of dried ghosts contained a large fraction of these "normal" ghosts. A separate study of Thalassemia major (Hoffman et al., '56) indicated a qualitative approach to the possible identification of host ghosts in this mixed cell population. The value given in table 1 is based on this method of "separation" and thus represents a maximum that one can expect since no HDP were found in the supposed "normal" ghosts.

The number of HDP per ghost is quite variable both in the human anemias studied and in the other mammalian species whose ghosts showed them. With the assumption that no HDP were lost during the procedure leading to the final dried ghost then the order given in table 1 is representative. The variability referable to the severity of the anemia is masked by this method of listing.

Isolation of these high density particles from whole blood was carried out by another technique. A 10 cm^3 sample of Thalassemia major blood was frozen at — 20° for 20 minutes and thawed at room temperature. This was repeated three times. This hemolyzed sample was then diluted with about one volume of isotonic saline, mixed and a portion centrifuged at 15,000 × g in the air turbine described by Parpart and Green ('51) for 15 minutes. This served to remove the ghosts. An aliquot of the supernatant was then centrifuged at 300,000 × g in a modified angle-head air turbine rotor (diameter 4.5 cm) for 40 minutes. The supernatant was removed and the invisible residue was washed once with distilled water. The residue was again resuspended in distilled water and a drop of this placed on a collodion screen and allowed to dry in the air. Figure 3 is a micrograph of such a preparation showing the HDP of the same dimension as seen before scattered over the collodion. An unknown lower density material is also present. Control blood prepared in a similar manner did not yield any HDP.

DISCUSSION

One of the immediate problems raised concerns the chemical identity of the HDP found in the ghosts isolated from the blood of certain erythrocytic anemias and species of mammals. These HDP, as yet, have not been obtained in sufficient quantity to permit direct chemical analysis. One reason for this difficulty can be ascertained by estimating the concentration in whole blood. If one makes reasonable assumptions as to the molecular size of the HDP, the number per cell and the number of cells containing HDP per liter, then the calculated concentration is of the order of 10^{-9} to 10^{-8} moles per liter. An attempt was made to obtain an electron diffraction pattern from the specimens prepared by the isolation procedure described above, but this yielded only a diffuse pattern characteristic of collodion alone. One can deduce, however, from consideration of the size of the particles and their intrinsic electron density that the metal content of each particle must be quite large.

Morphologically the HDP bear a certain resemblance to ferritin, an iron storage compound containing 17 to 23% iron (cf. Granick, '51). Farrant ('54) has studied with the electron microscope ferritin prepared from horse spleen involving crystallization with $CdSO_4$. The ferritin molecules (iron micelles) described by Farrant possessed a 4-fold symmetry which has not been observed in our preparations. On the other hand, the HDP are of similar dimension, electron density and shape as indeed are the majority of ferritin molecules in the unshadowed micrograph given in Farrant ('54). We must conclude that until additional evidence is obtained the morphological features of the HDP alone are insufficient to establish an identity with ferritin. According to Granick ('51) ferritin has not been found in whole blood (except in the horse), but is present in bone marrow.

Emphasis should be placed on the intracellular localization of the HDP. They could only be obtained free from the intact cell or ghost by fragmentation with freeze-thawing. The holes thus produced are large enough so that the HDP in addition to hemoglobin can be washed out of the cell or ghost in an isotonic solution. HDP have not been found in the hemolysate obtained by hypotonic hemolysis but are retained by the ghosts. Thus, the conclusion is drawn that the HDP are on the inside of the intact cell and that osmotic hemolysis produces no holes as large as the HDP. This is perhaps anticipated in view of the reconstitution phenomena exhibited by osmotically prepared ghosts (Hoffman, '54).

Of considerable interest is the fact that in humans the HDP appear to be restricted to the class of hereditary red cell dyscrasias with the possible exception of pernicious anemia. The presence of HDP in the one case of ovalocytosis examined is not an exception since this disease is genetically transmitted; also interesting is that this patient is the aunt of a patient with Thalassemia major. The results, however, within the human anemias examined seem to be diagnostically characteristic. Whether this approach should prove fruitful as a diagnostic tool will have to await further study.

Discussion of the possible significance of HDP would at best be speculative without additional knowledge of their composition and a more quantitative determination of their distribution.

SUMMARY

1. New high density particles (HDP) have been found in the isolated red cell ghosts from humans with the following types of anemias: Thalassemia major and minor, sickle cell disease and carrier, erythroblastosis fetalis, congenital hemolytic icterus, ovalocytosis and pernicious anemia.

2. HDP have not been observed in the ghosts from normal humans, nutritional anemias, polycythemia vera, lymphocytic leukemia or in the anemia of lead poisoning.

3. HDP are present in bat, camel, beef, mouse, pig and sheep ghosts but not in those from the groundhog, rat, and rabbit.

4. HDP vary in size from 40 to 100 A and appear to be present in the cell interior. The number of HDP in a single ghost and in the proportion of ghosts containing them in any one sample of blood was found to vary markedly. HDP have been isolated but not identified.

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PLATES

PLATE 1

EXPLANATION OF FIGURE

1 **Magnification 100,000 ×. The edge of this air-dried untreated ghost is on** the lower left. Within the ghost area high density particles (HDP) ca. 100 A in diameter can be seen. The total number of HDP occurring in this ghost is between 10,000 and 100,000. This ghost was isolated from the blood of a patient **with Thalassemia major. (7001 c).**

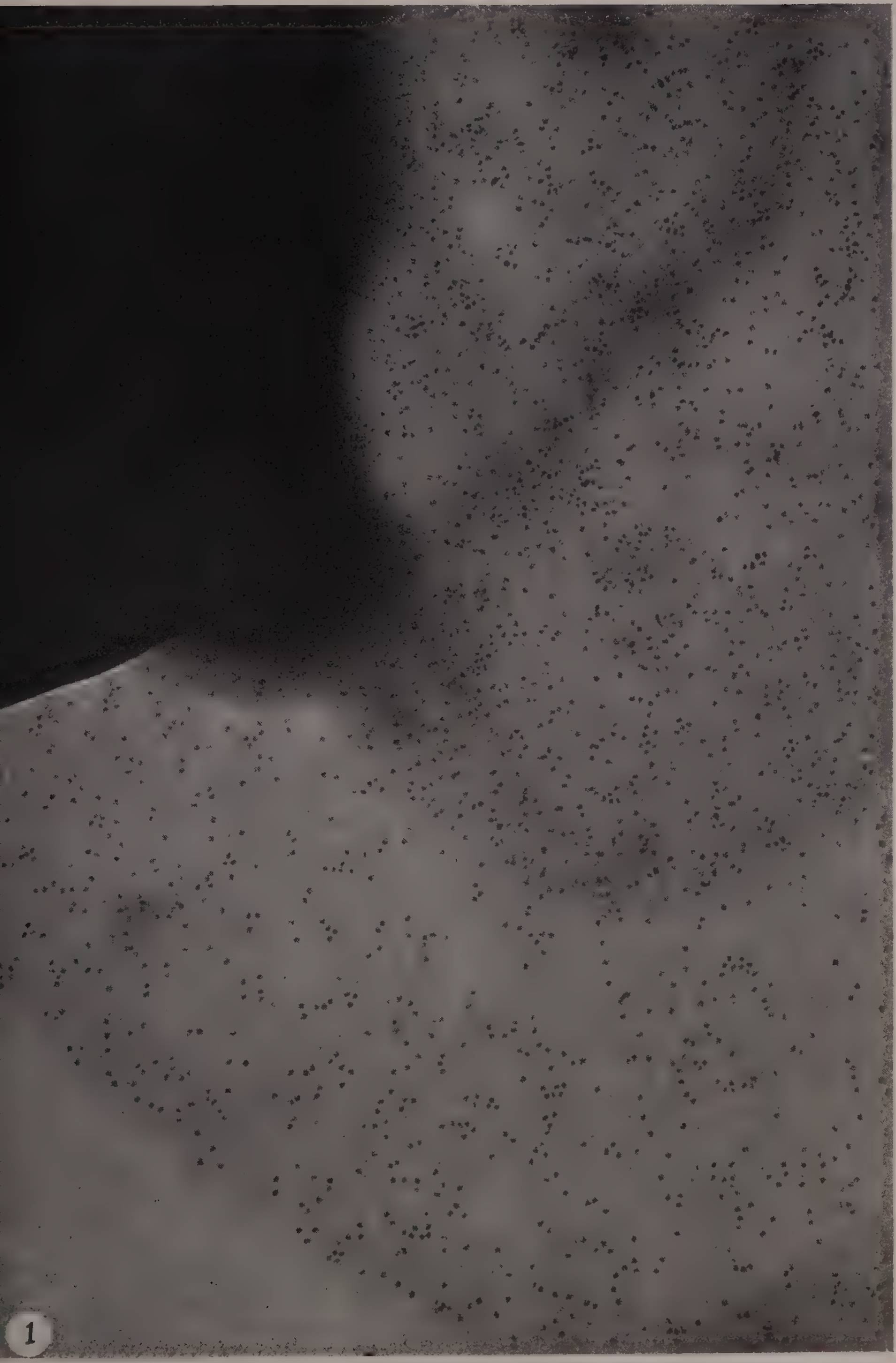
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PLATE 2

EXPLANATION OF FIGURE

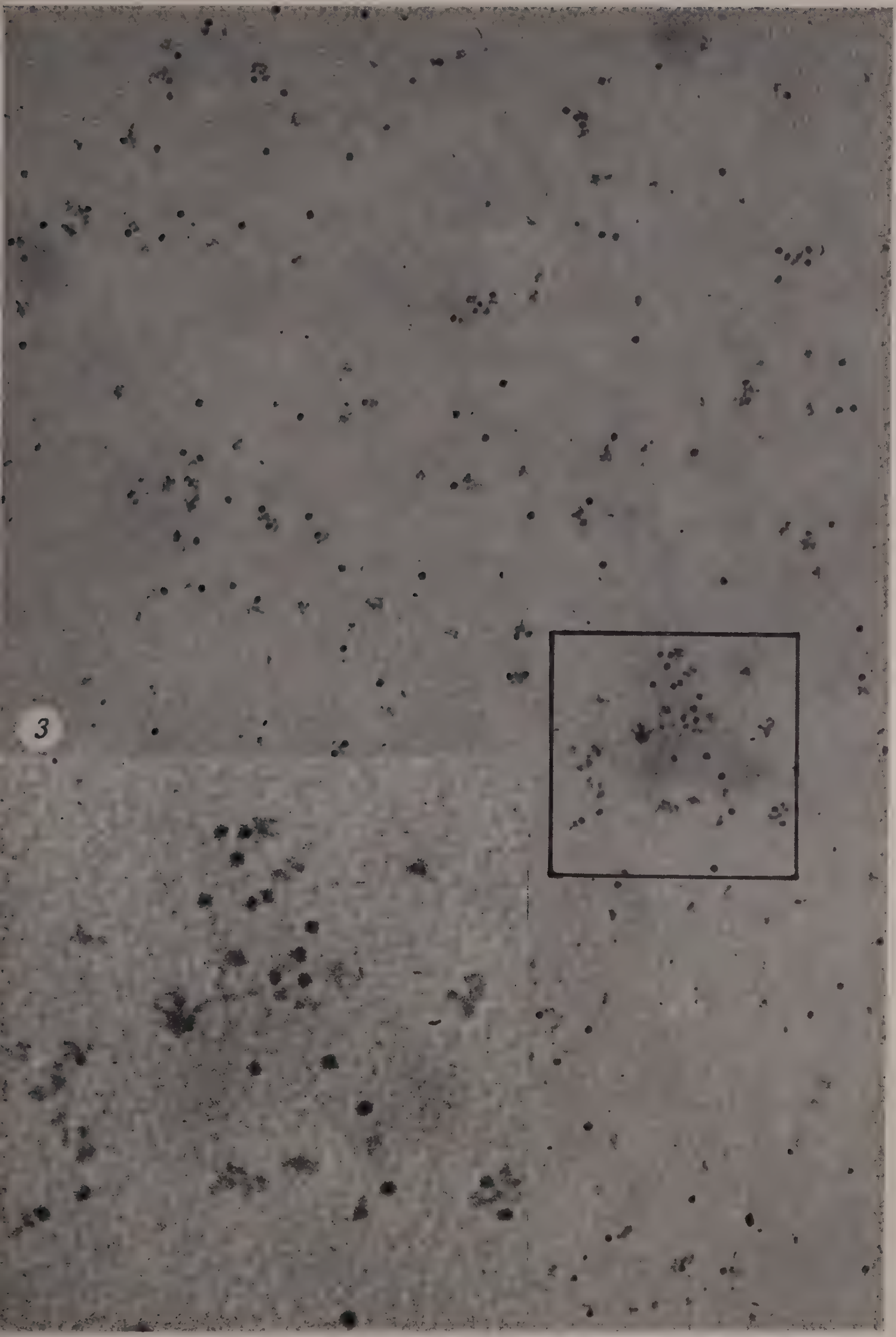
2 Magnification 100,000 ×. This ghost was treated with phosphotungstic acid prior to air drying (separate experiments showed that the appearance of the particles is unchanged by this treatment). The HDP vary in size from 40 to 100 A and are seen over the whole ghost. The total number of HDP in this ghost lies between 10,000 and 100,000. This ghost was isolated from the blood of another patient with Thalassemia major. (7065 b).

2

PLATE 3

EXPLANATION OF FIGURE

3 Magnification 100,000 ×. These are HDP isolated from a sample of whole blood from a patient with Thalamessia major by the procedure described in the text. The HDP are ca. 100 A in diameter. The inset is a higher magnification (210,000 ×) of the outlined area. (7155 b).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ON THE REPRODUCIBILITY IN THE OBSERVED ULTRASTRUCTURE OF THE NORMAL MAMMALIAN RED CELL PLASMA MEMBRANE [1]

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Department of Biology, Princeton University, Princeton, New Jersey

THIRTY FIGURES

INTRODUCTION

The surface of the isolated plasma membrane as observed with the electron microscope was interpreted by Hillier and Hoffman ('53) to be composed of plaques connected to an underlying tangential fibrous component. This plaque structure was found to be characteristic of all the ghosts that were examined. If looked for, plaques can also be discerned, though less clearly, in the published results of other workers of similar preparations (cf. Hillier and Hoffman, '53, for references). Coman and Anderson ('55) using a carbon-replica technique have observed that the shadowed dried surface of the intact erythrocyte likewise displays plaques. However, there has been no systematic investigation of the variability of the plaque structure of ghosts under controlled conditions.

This study describes certain experiments designed to test the reproducibility of the observable surface structure of ghosts prepared in a standard manner. The results indicate that within a given experiment the plaque structure varies very little beyond certain defined limits. On the other hand, the comparison of different experiments with each other shows a qualitative variation not in kind but in degree. However, advantage is taken of the reproducibility found in a given

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[1] This work was supported in part by the Whitehall Foundation.

experiment to study the surface structures of several mammalian species. The work of Jacobs ('50) and Jacobs, Glassman and Parpart ('50) shows that the permeability properties of red cells from different mammals are characteristic for each species. This would imply that the surface structure of the plasma membranes of various species should reflect at a molecular level these physiological differences. That differences were, in fact,observed will be illustrated below. However, the relationship of this observed structure to that of the intact unaltered membrane is not elucidated by these experiments.

METHODS AND MATERIALS

All ghosts used in this study were prepared for electron microscopy by the method of successive hemolysis as outlined by Hillier and Hoffman ('53) incorporating the modifications given in Hoffman et al. ('56a). The contamination-free ghosts were mounted on collodion while wet and then exposed to 0.1% phosphotungstic acid (PTA), washed in redistilled water and dried in air. After air drying, the ghosts were shadowed with chromium (Williams and Wykoff, '44) and examined, at an electron optical magnification of 15,400 ×, in an RCA type EMU-2A electron microscope using a high resolution double objective.

The methods used to collect the samples of blood from the various species were the same as those described in Hoffman et al. ('56a). The magnification of all illustrations is 75,000 ×.

RESULTS

The length of exposure to 0.1% phosphotungstic acid previously used by Hillier and Hoffman ('53) was three minutes. To determine whether this time gave adequate fixation the ghosts were exposed in a separate experiment for 0 (the control), 1, 3, 5 and 15 minutes. The results shown in plate 1 are representative. If the ability to differentiate plaques is taken as an index of adequate fixation it appears that one minute is not long enough. On the other hand, exposure to PTA for any length of time between 3 and 15 minutes seems sufficient for fixation since the results are indistinguishable from each other. Thus, as a matter of convenience, three minutes was adopted for the length of exposure and was used throughout as a standard part of the preparative procedure. The uniformity of the results of this experiment gives evidence of the reproducibility of the preparative procedure.

The normal human blood used in this study was collected drop by drop from the finger of one individual (JFH). Routinely drops 2 and 3 were collected into a small volume of a citrate-saline solution and immediately put through the preparative procedure. To test whether this method of sampling the total blood volume was adequate an experiment was performed in which drops 2 and 3, 6 and 7, 13 and 14, and 21 and 22 were prepared separately but simultaneously and under conditions as identical as possible. Plate 2 shows representative pictures of each of these preparations. The amount of variation apparent in the surface texture in these selected populations of cells is considered to be minimal. In addition, this experiment provides evidence that the separate preparation of 4 samples of blood does not introduce variation greater than that which would be expected from pooling the 4 samples and preparing the mixture as a whole. The fact that the ghosts of these various drops of blood appear indistinguishable argues that the use of drops 2 and 3 yields a preparation which is probably characteristic of what would be expected experimentally of the total circulating red blood cells.

It is of interest to describe the general method of determining which micrograph is selected for illustration (subsequently termed ‘‘representative’’) in the various plates. Twenty to 50 electron micrographs are usually taken of each preparation of ghosts. All pictures are printed and then evaluated with regard to the appearance of the surface textures. Subjectivity is minimized but not eliminated by the appraisal of several individuals in a like manner. The results of this procedure uniformly indicate that approximately 90% of the pictures were almost indistinguishable from each other — that is, they *looked* alike. The remainder were obviously different and were considered to represent extremes. Indistinguishable as used in the present context is relative only to a certain level of evaluation. No method is known for describing in a quantitative fashion the structures obtained in this study. Therefore, to convey clearly the level of organization being referred to becomes a complex problem. In attempting to understand what the components at this level are, they have been interpreted in terms of the plaque theory. Thus ''surface texture'' is equated to plaque size, shape and arrangement. The information content of these electron micrographs surely ranges beyond this, but for the present this degree of abstraction appears to be adequate.

In plate 3 are shown 5 additional micrographs of the same preparation as in figure 6. Figure 14 represents the greatest amount of variation from the other 4. The selection of the ''representative'' picture (used in plate 2) is based not only on the evaluation of the surface texture but also on the electron optical focus and essentially amounts to an arbitrary choice.

In order to determine if the time of withdrawal of blood from an individual during the day and whether incubation at room temperature of the blood influenced the observed surface texture the following experiment was performed: Initially (zero time) drops 3 through 13 were collected and a portion (drops 6, 7, 8) immediately prepared for electron microscopy. At the end of 4 hours a fresh sample of blood (drops 6, 7, 8) was again obtained, split, and one portion of this together with a second aliquot of the zero sample was again prepared. Four hours later (8 hours from zero time) another sample of fresh blood (drops 6, 7, 8) was collected and prepared along with a third aliquot of the zero sample and the second aliquot of the 4 hour sample. This procedure allows three types of comparison: (a) the initial samples of each representing zero, 4 and 8 hours; (b) the zero and 4 hour sample for 4 and 8 hours and for 4 hours respectively; and (c) the initial and subsequent samples with each other. Plates 4 and 5 are representative of the results of this experiment.

It is seen that no readily observable differences are apparent. This is interpreted to mean that the structure of the plasma membrane is relatively constant under these conditions and independent of the time during the day a sample is removed for study. It also does not seem of primary importance to prepare the blood immediately since incubation at least for 8 hours at room temperature (ca. 22°C.) appears ineffective in altering the structure. In addition, the results provide further evidence of the reproducibility of the preparative procedure.

Plate 5 shows a reticulocyte from one of the samples in the experiment above. When this is compared with plate 4 and the other illustrations on plate 5, it becomes obvious that the *surface texture* is the same as that of the older cells. This result has been a consistent finding in both normal and abnormal blood where the blood picture has not been altered with transfusion. This fact was found helpful in the characterization of the ghost surface in samples of blood from patients with Thalassemia major (Hoffman et al., '56b).

The identification of the ghosts of reticulocytes (as illustrated in plate 5) was based initially upon the correlation of the appearance of the intact cell and its ghost in *wet* mounts using the television microscope (Parpart, '51). Under this circumstance intracellular particles can be resolved and appear in brownian motion in the interior of the intact cell. Upon hemolysis, the number of visible particles is increased in addition to the enhancement of particle contrast. The reticulocyte count of the intact population and the number of wet ghosts containing particles are comparable. No particles resolvable by the light microscope are normally seen in mature human cells. Thus, ghosts of reticulocytes contain particles. Next, the proportion of ghosts examined in the electron microscope, displaying gross particles is closely correlated with the reticulocyte count of the initial whole blood sample. This fact supports the idea that the ghosts lost during the preparation were indiscriminately selected. Further, in certain blood dyscrasias such as sickle cell anemia, Thalassemia major and pernicious anemia where the reticulocyte count is higher, the number of particle- containing ghosts is proportionally increased. Lastly, the isolation of reticulocytes by centrifugation (cf. Chalfin, '56; Keitel et al., '55) also yields ghosts rich with gross particles as seen in plate 5.

Illustrated in plates 6 and 7 are representative ghosts prepared from the blood of the mouse, pig, camel[2] and ox. It is immediately apparent that the surface textures are distinctly different from each other and from human. These species are arranged in the order of plaque size from large to small (figs. 23, 24, 25, and 26 respectively). This sequence in reverse emphasizes the level of ''compactness'' of the surface texture. In every instance the blood of these species was prepared along with human blood. Given the reproducibility of the preparative technique, as indicated above, the character of the surface textures represented by these species is markedly different from that ever observed in human ghosts. Therefore, the assumption appears valid that species vary in the molecular structure of their plasma membranes.

DISCUSSION

From the experiments reported above the conclusion may be drawn that the preparative technique yields for a particular preparation ghosts which appear to have essentially the same surface texture. This reproducibility makes possible the interpretation and evaluation of various manipulations introduced during a single experiment as was done by Hillier and Hoffman ('53). In their paper, figures 1, 7, 16, 17, 18, 25 and 29 and figures 13, 14 and 15 represent two different samples of ghosts prepared from the blood of the same individual (JFH) on separate occasions. These two groups of representative electron micrographs show that when ghosts are subjected to a variety of experimental treatments (at the same time) the resulting differences can be ascribed to and are introduced by the treatment per se, for the observed variation lies outside that which can be accounted for by the preparative procedure.

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[2] Provided through the kindness of Dr. Charles P. Gandal of the New York Zoological Society.

The question can now be raised: what is the day to day reproducibility of the preparative technique using blood from one individual? The ghosts in plate 1 and plates 2 and 3 were prepared on January 20 and 21, 1953, respectively; those in plates 4 and 5 at three different times on January 26, 1953; Figure 7 and plate 5 of Hillier and Hoffman ('53) on February 26 and March 6, 1952, respectively. Illustrated in plate 8 are representative pictures of comparable preparations made on November 5 and 20, 1952; January 23, 1953; and June 4, 1953 (figs. 27, 28, 29 and 30, respectively). When the results of these separate experiments are compared with each other it is seen that there is a moderate amount of variation from day to day in the surface textures and that this variation is greater than that expected from any one experiment. There are three possible explanations for these differences: (1) the ghosts themselves (and the cells from which they were derived) are different, (2) the preparative procedure was not reproducible, or (3) a combination of both. The fact that the preparation of a number of different blood samples from different species consistently yield results that are different from each other in a reproducible manner supports the third alternative (in addition see Hoffman et al., '56b). However, further work is necessary to determine the extent to which possibilities (1) and (2) are expressed under the present experimental conditions.

From an interpretation of the data found on the length of life of human red cells (Ashby, '48) it is possible that in time the surface of the intact cell might change thereby signalling its uptake by the reticulo-endothelial system. If one assumes that the ghosts are distributed randomly with respect to age on the final collodion supporting membrane and since there was no selection during the preparation (approximately 90% recovery of the beginning number) and that the majority of ghosts appear to have similar surface ultrastructures, with no significant differences between mature and reticulocyte, then it follows that no correlation of the plaque structure with age is possible within the resolution limit of 20 A and under the experimental conditions used in this study. The membranes observed that fall outside the "representative" group could possibly represent terminal differences though this interpretation requires further study.

The evidence presented in plates 6 and 7 of this paper and figures 30 and 31 of Hillier and Hoffman ('53) strongly suggest that the molecular organization of the plasma membrane is distinct for each species in addition to being different from man. That is, the preparative procedure induces a response in each type of ghost dependent upon its molecular composition. The intrinsic features which must be responsible for these differences are not at present understood and emphasize the problem of relating the structures observed with the electron microscope to the physiology of the intact cell.

In the light of these species' differences it is of interest to consider the implications of the plaque model outlined by Hillier and Hoffman ('53). It was postulated by them that the "pore" would be the area between the plaques. For the case where plaques are packed in an hexagonal array the total pore area is independent of the plaque size though the pore size would vary as a function of the plaque size. This means that permeabilities of different types of red cells can be compared, as ordinarily done, since the amount of permeable surface area would be unity in all instances. Thus, if the species studied are arranged in order of decreasing *plaque size* the following sequence is obtained:

Groundhog, mouse > rat, human, pig > camel, ox.

The relative permeability of the red cells of these species to three non-electrolytes, from the data of Jacobs, Glassman and Parpart ('35, '50), is given in table 1. If the permeability to thiourea is used as a basis of comparison there appears to be a correlation of plaque size and permeability. However, comparison of this sequence to the relative permeabilities of ethylene glycol and glycerol obviously does not show the same correspondence. It could be argued that the exceptions (rat and human) to this alignment are those same cells which have an abnormally high permeability to glycerol and which can be altered by the addition of traces of copper. There is some evidence that the permeability to ethylene glycol also is unusual in these same species (Jacobs, '54). Since there is not yet enough data available both on the electron microscopy of different species and their permeability to a variety of substances no conclusion can be drawn concerning the correlation of plaque size and permeability. However, there are other considerations which indicate that this situation is still more complex.

The manner in which the surface texture of the human ghost (and this applies to other species as well) is seen to vary from day to day can be described as changes essentially in plaque size, with minimal alteration in the arrangement of plaques. On the other hand, species seem to differ from each other both in plaque size *and* in arrangement. Plaque shape does not appear to play an important role in the characterization of differences. Thus, this implies another parameter operating which must be taken into account in the interpretation of the species differences in terms of the plaque model.

TABLE 1

The time (in seconds) of hemolysis in 0.3 M solutions of non-electrolytes at 20°C.

	GROUND-HOG	MOUSE	RAT	HUMAN	PIG	CAMEL	OX
Thiourea	3	20	44.3	104	. . .	. .	183
Ethylene glycol	1.9	5.7	3.6	4.2	15.6	. .	27.4
Glycerol	2.2	44.9	18.2	32.2	1316	. .	2169

The analysis of the chemical composition of the ghosts of a number of species of mammals as done by Parpart and Dziemian ('40) indicates that the total lipid content is about the same for all species examined. The relative proportions of various types of lipids was also not significantly different, but the ratio of lipid to protein was found to vary. How this enters into the interpretation of the electron micrographs is not at all clear. It can be said, however, that the observable differences in the surface textures probably represent a combination of the same molecules (lipid) and different molecules (protein) assembled differently. The site and orientation of each type of molecule must be determined before the basic structural configuration of the plasma membrane will be satisfactorily explained.

SUMMARY

Ghosts of human erythrocytes prepared in a standard manner were examined with the electron microscope. The reproducibility of the surface texture was studied using blood obtained from one individual. It was found that in any one sample of blood the amount of variation was small: approximately 90% of the ghosts appear indistinguishable. Ghosts prepared from blood collected at different times on the same day were likewise indistinguishable from each other. However, when ghosts which had been prepared on different days were compared the variation in the surface textures were greater than that observed in any one sample. Factors responsible for these differences are indicated. The reproducibility of the preparative procedure for any one day gave a means of evaluating the surface textures of ghosts derived from different species. It was found that the surface ultrastructure of the ghosts of man, mouse, pig, camel and ox were characteristically different from each other. These results provide evidence that the known physiological differences have a molecular morphological basis. The significance of the findings is discussed with reference to the plaque theory.

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PLATE 1

EXPLANATION OF FIGURES

This plate illustrates the effect of length of exposure to 0.1% phosphotungstic acid on the plaque structure of human erythrocyte ghosts. Magnification 75,000 ×. Shadowed. See text for interpretation.

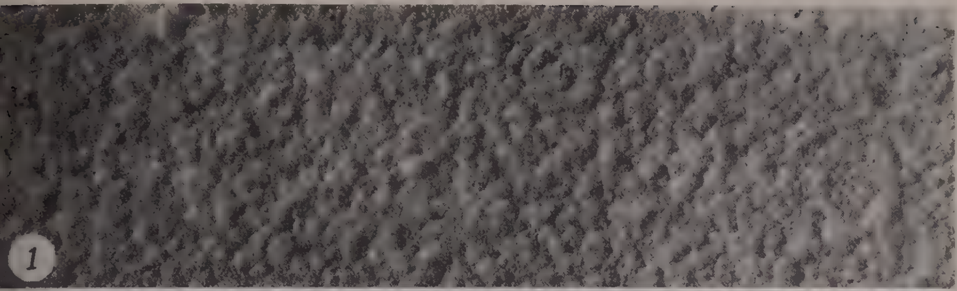
1 This is the control unexposed specimen. (1094c)

2 One minute exposure. (6669b)

3 Three minute exposure. (6674e)

4 Five minute exposure. (6678d)

5 Fifteen minute exposure. (6684a)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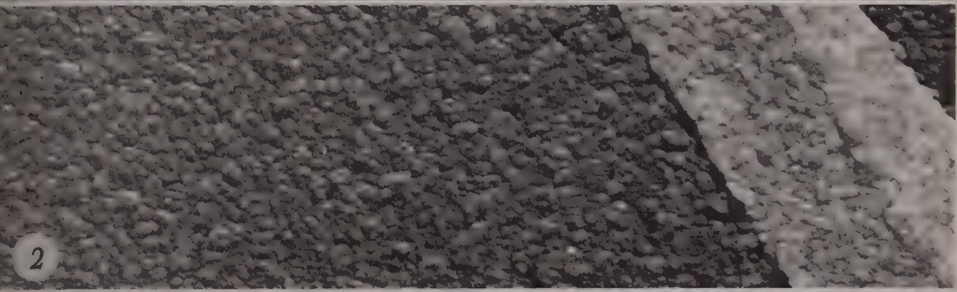
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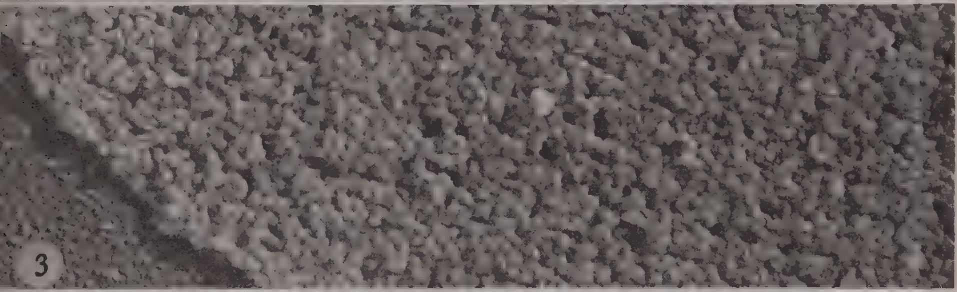
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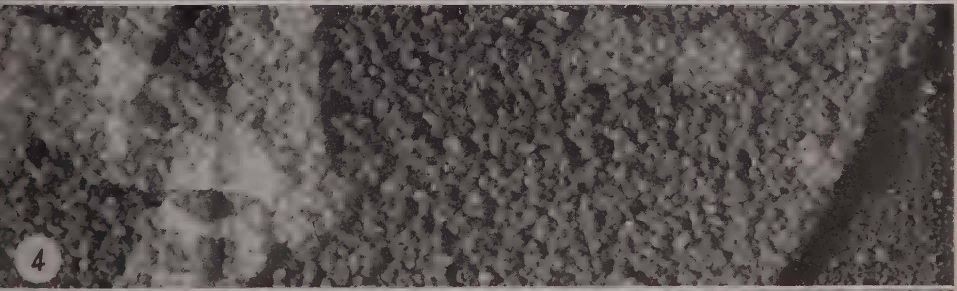
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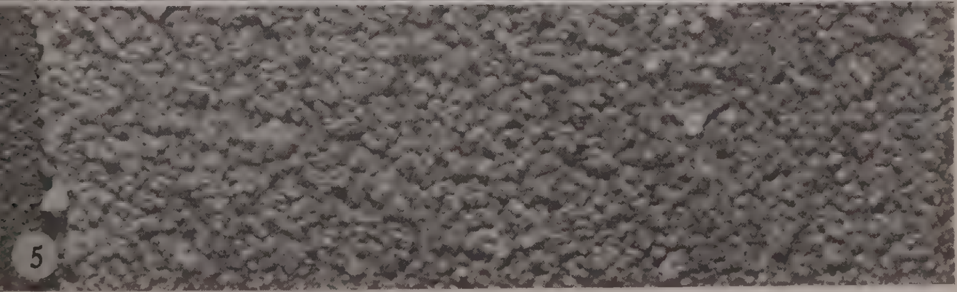
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PLATE 2

EXPLANATION OF FIGURES

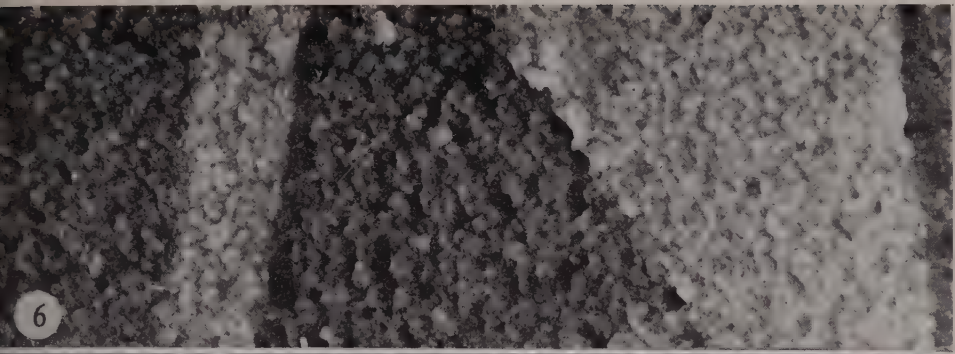
The ghosts of human red cells shown in this plate are from selected portions of blood emanating from a finger puncture. The ghosts were prepared separately but under closely identical conditions. When these preparations are compared it becomes apparent that the plaque structures are indistinguishable from each other. Magnification 75,000 ×. Shadowed.

6 Representative ghost from drops 2 and 3. (6701e)

7 Representative ghost from drops 6 and 7. (6705d)

8 Representative ghost from drops 12 and 13. (6707e)

9 Representative ghost from drops 21 and 22. (6711e)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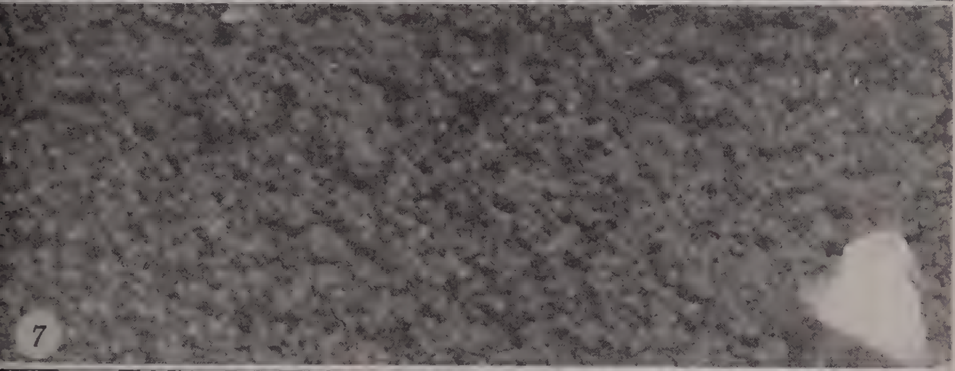
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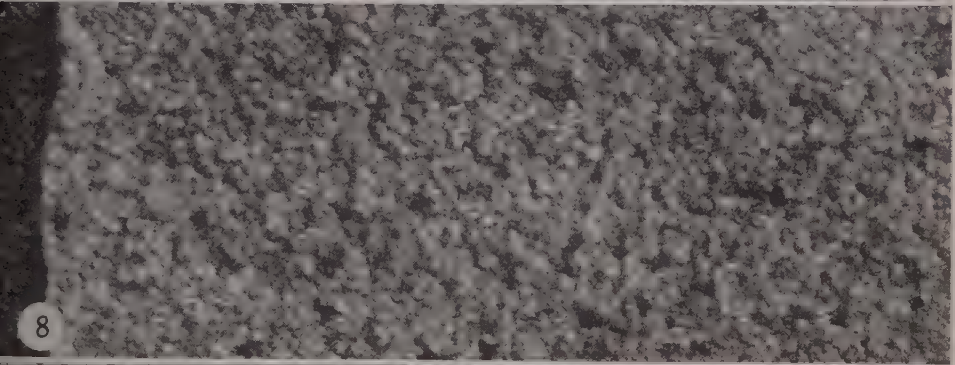
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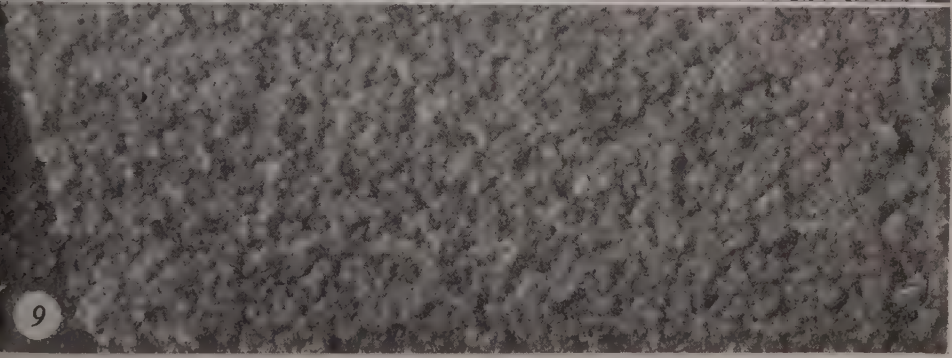
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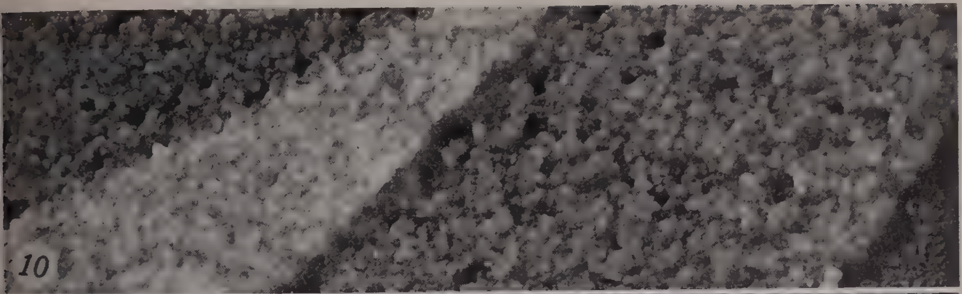
PLATE 3

EXPLANATION OF FIGURES

The ghosts shown in this plate are five additional micrographs of the same sample of human blood from which figure 6 was derived. The purpose of this plate is to illustrate the basis for the selection of the micrograph which is ''representative'' of the results. Approximately 90% of the ghosts appear indistinguishable as evidenced by the comparison of figures 10, 11, 12, 13 and 6; the ghost shown in figure 14 has a different surface texture from the others and is characteristic of the type of variation which compose the remaining 10%. See text for a more extensive discussion. Magnification 75,000 ×. Shadowed. (6702c, 6701a, 6701b, 6700d, 6700e).



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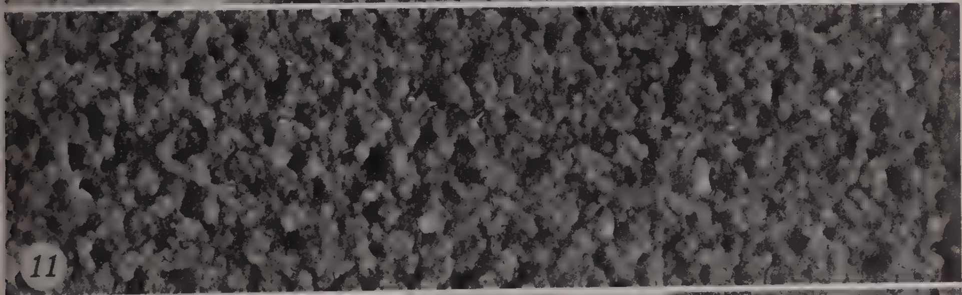

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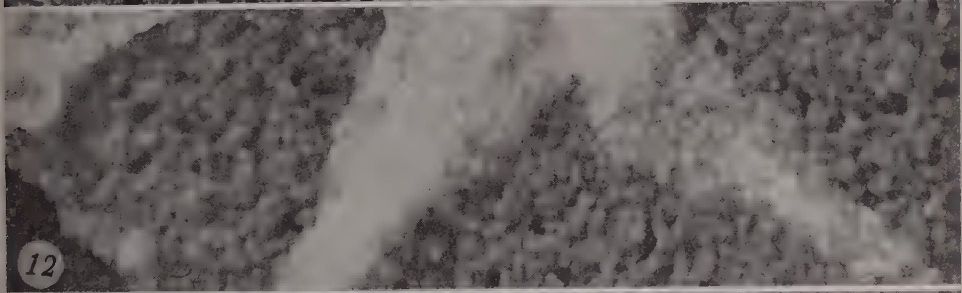

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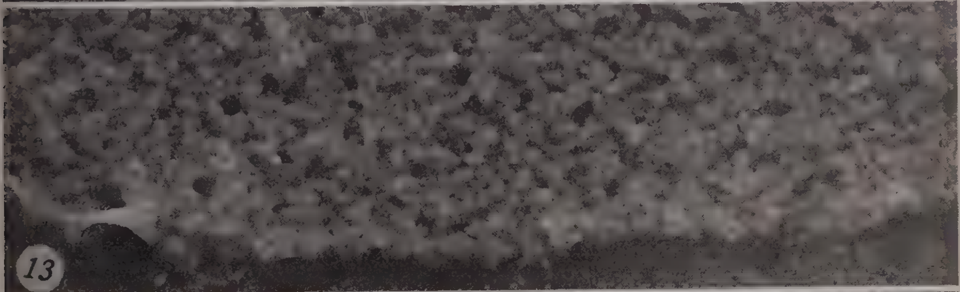

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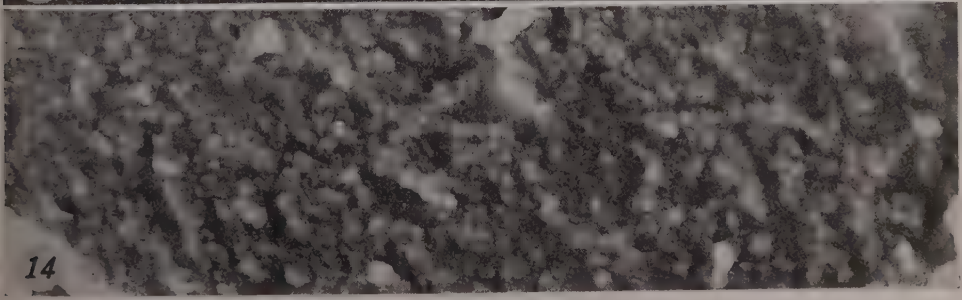

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PLATE 4

EXPLANATION OF FIGURES

The results of the following experiment on human blood appear on plates 4 and 5. Three samples (1, 2 and 3) of blood were taken at three different times (A, B and C) from one individual on the same day. A equals zero time, B equals 4 hours and C equals 8 hours. Ghosts were prepared immediately after each collection of blood and except for sample 1 at time A included aliquots of all samples collected prior to that time. Thus, sample 1 was prepared thrice; sample 2, twice, and sample 3, once. The results illustrate the reproducibility of the preparative procedure. Each ghost shown is representative of that particular preparation. Magnification 75,000 ×. Shadowed.

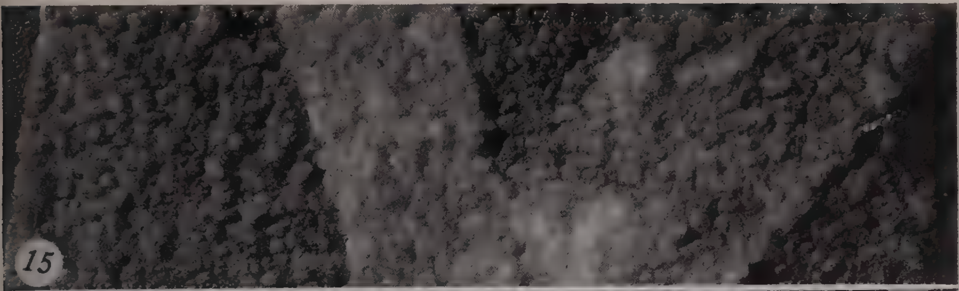
15 Sample 1 a (drops 6, 7, 8) collected and prepared at time A. (6718a).

16 Sample 1 b (drops 2 through 5, 9 through 13) collected and prepared at time A. (6723a).

17 Sample 1 b prepared at time B. (6806c).

18 Sample 2 collected and prepared at time B. (6809a).

19 Sample 1 b prepared at time C. (6814d).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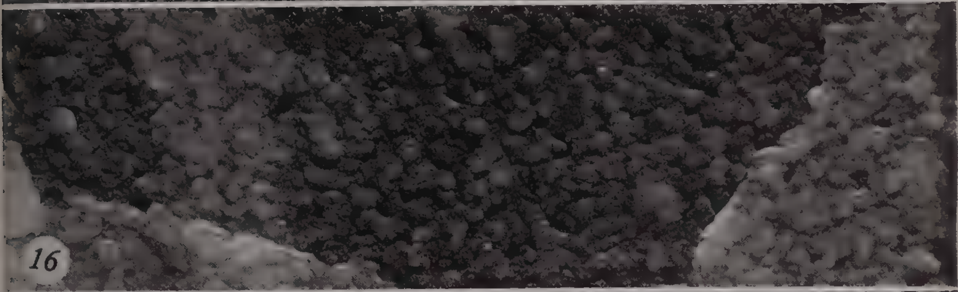
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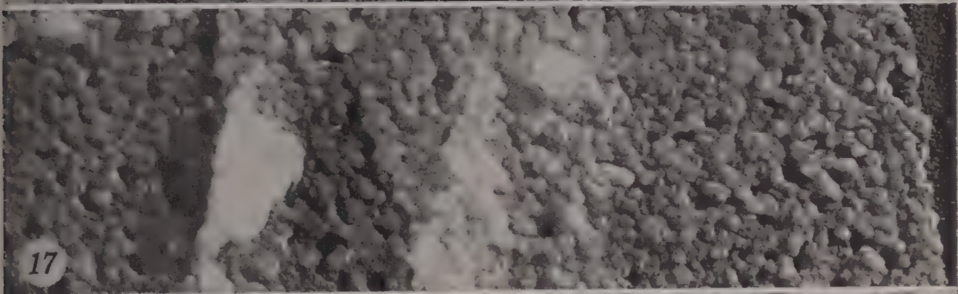
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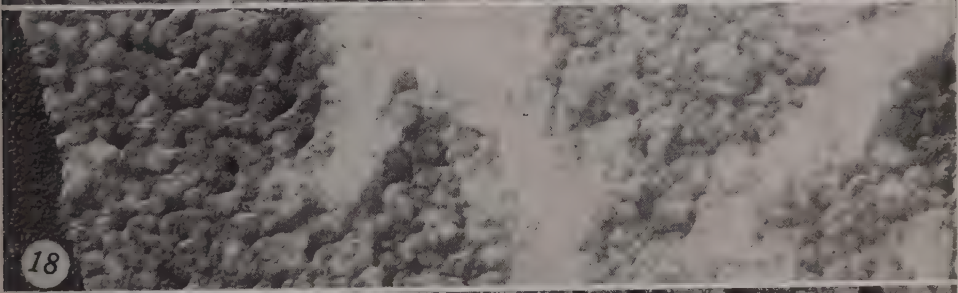
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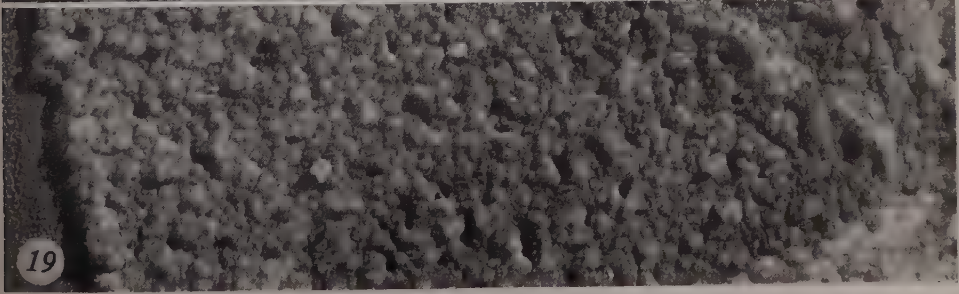
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PLATE 5

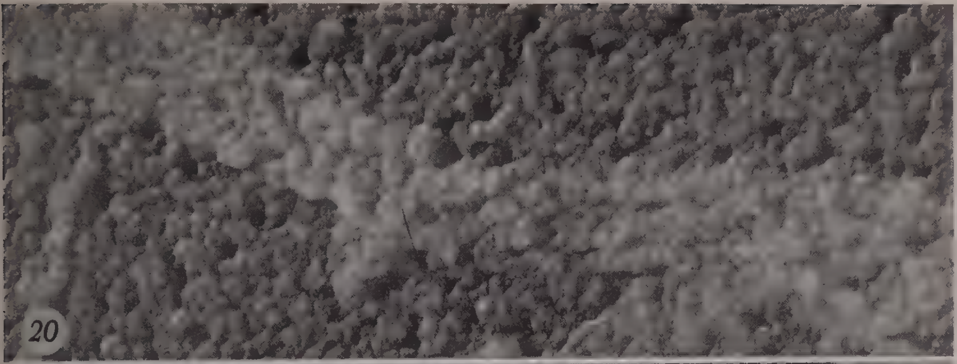
EXPLANATION OF FIGURES

The legend of plate 4 describes the experiment concerning the figures shown on this plate. Magnification 75,000 ×. Shadowed.

20 Sample 2 prepared at time C. (6815c).

21 Sample 3 collected and prepared at time C. (6819e).

22 This is the ghost of a reticulocyte from the same preparation presented in figure 19. The basis for the identification as a reticulocyte is considered in the text. That the surface textures of the ghosts of reticulocytes and mature cells are indistinguishable is shown by comparing this figure with figure 19 (6814b).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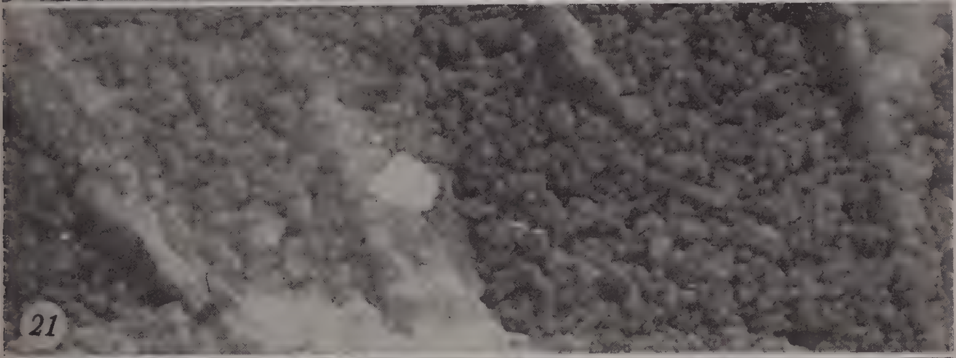
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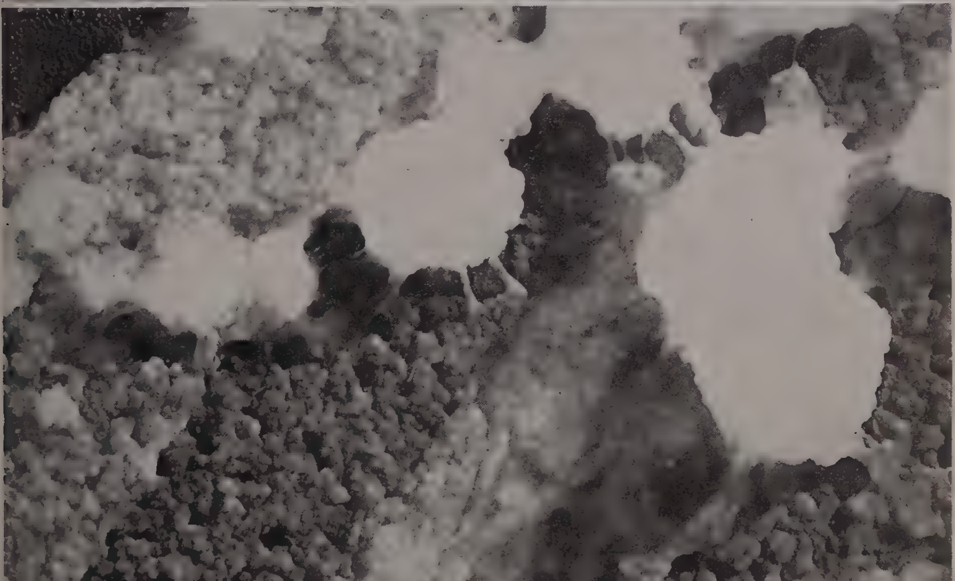
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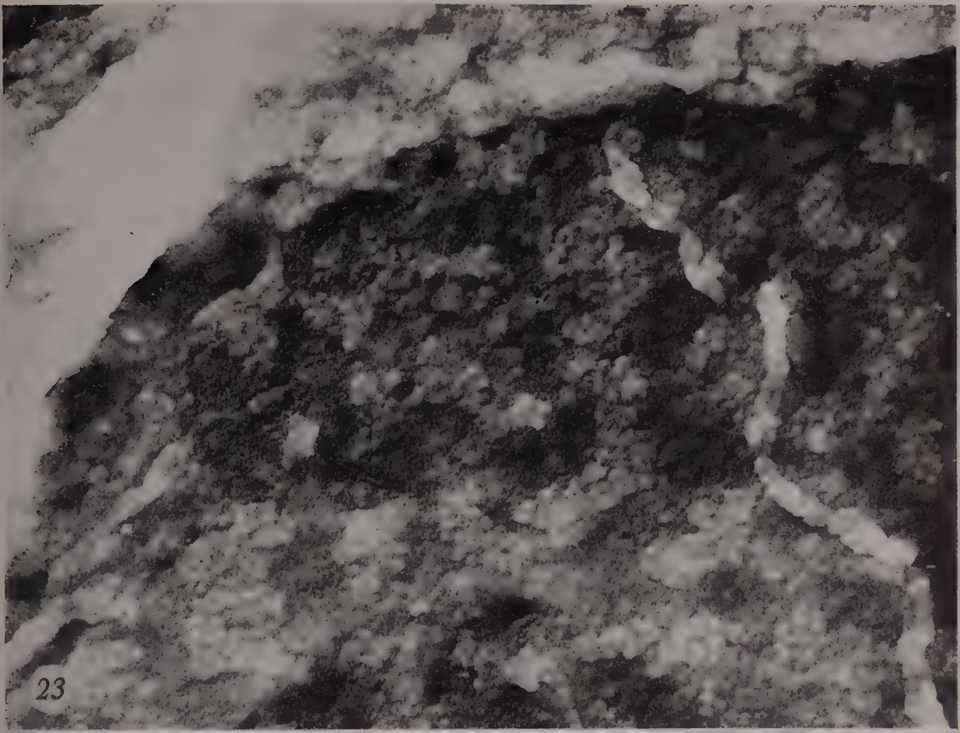
PLATE 6

EXPLANATION OF FIGURES

23 Representative ghost from a sample of mouse blood. Magnification 75,000 ×. Shadowed. See text (7448c).

24 Representative ghost from a sample of pig blood. Magnification 75,000 ×. Shadowed. See text.



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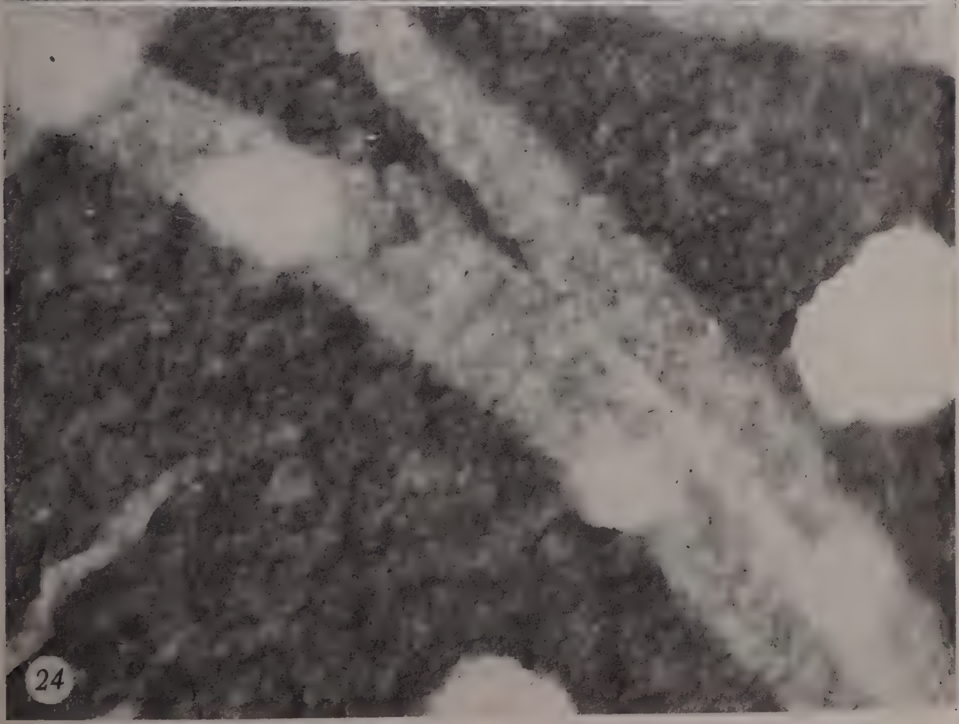

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PLATE 7

EXPLANATION OF FIGURES

25 Representative ghost from a sample of camel blood. Magnification 75,000 ×. Shadowed. See text.

26 Ghost from a sample of ox blood. The majority of ghosts in any one sample of blood display a surface texture similar to this ghost. However, the other portion of ghosts have a more definite identifiable plaque structure. The very dense (white) granules are called high density particles (HDP) by Hoffman et al. ('56a) but have a different form than those of all other species of mammals examined. Magnification 75,000 ×. Shadowed.

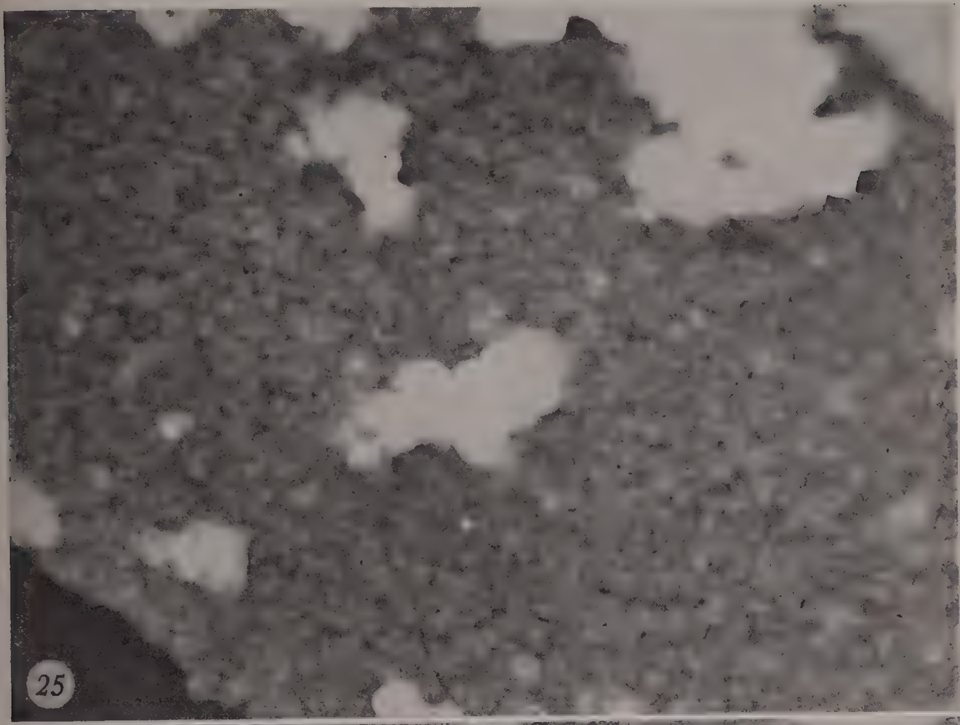
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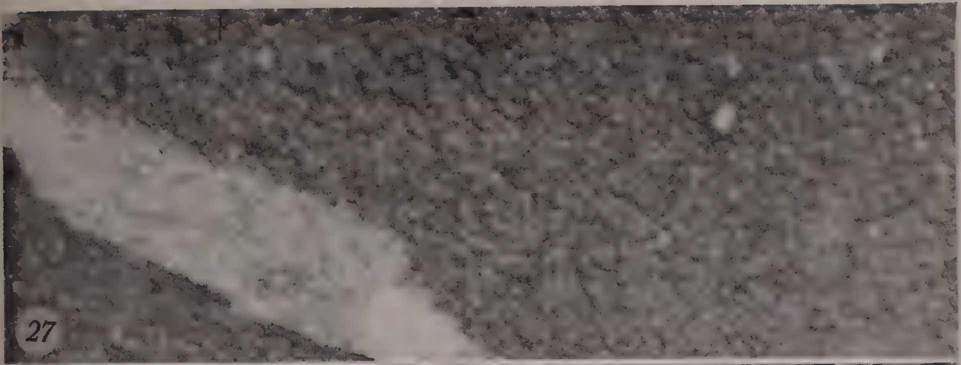
PLATE 8

EXPLANATION OF FIGURES

27, 28, 29 and 30 These are representative ghosts from samples of human blood collected and prepared on different days. Blood from the same individual used for this study was the same as for the experiments represented on all other plates excluding plates 6 and 7. Comparison shows that the preparations of blood on separate days yields similar results but that the variation among these samples is greater than that observed in a single preparation on any one day. Magnification 75,000 ×. Shadowed (6586c, 6618d, 6813b, 1114b).



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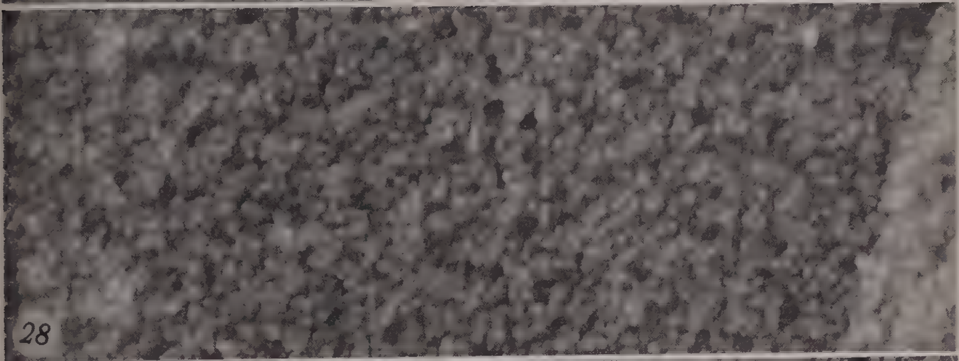

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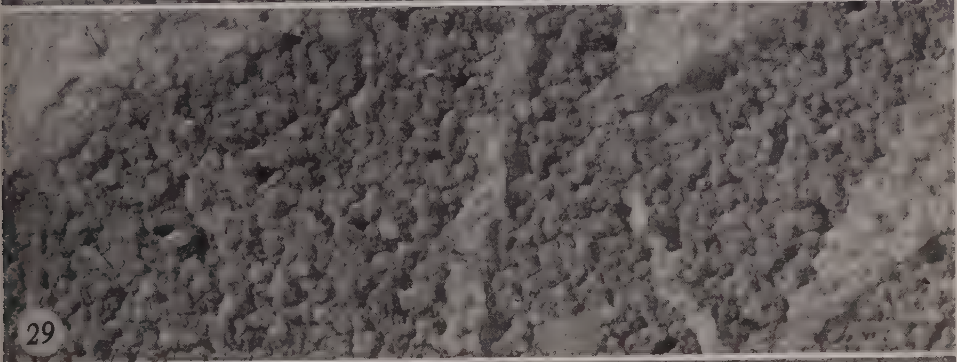

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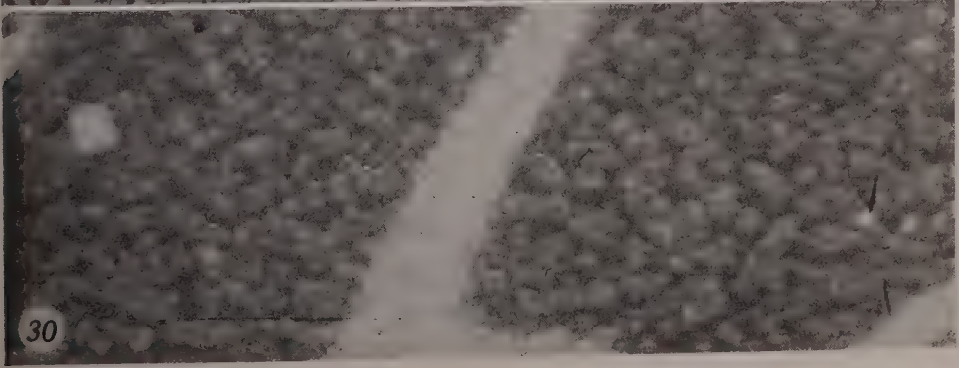

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THE EFFECT OF INCREASED PRESSURES OF OXYGEN UPON THE LUMINESCENCE OF ACHROMOBACTER FISCHERI

CHARLOTTE HAYWOOD, HAROLD C. HARDENBERG, JR.
AND E. NEWTON HARVEY
*Marine Biological Laboratory, Woods Hole, the Department of Physiology,
Mount Holyoke College, and the Department of Biology,
Princeton University*

TWO FIGURES

Although oxygen toxicity for living material has long been recognized (Bean, '45), the prevailing view that an enzyme inactivation is involved (Stadie et al., '44; Gerschman et al., '54) adds to its current interest. Hastings ('52) has noted that bioluminescence of Achromobacter fischeri is decreased reversibly in 100% oxygen at atmospheric pressure. The present study deals with this sensitiveness of luminous bacteria, as measured by alterations in light production, to oxygen at still higher pressures.

The bacterium, Achromobacter fischeri, Culture no. 7744 from the American Type Culture Association, was used for most of these experiments; for the others a culture obtained directly from Dr. F. H. Johnson was employed. Both cultures were presumably from the same strain. In setting up an experiment, bacteria from an agar plate were suspended in "PN solution" (1 part, mixture of M/2 NaCl + 1% glucose; 1 part, M/4 phosphate buffer at pH 7.3) (Harvey, '52). Fifteen to 20 drops of this suspension were placed in a 1 oz. shell vial which was inserted in a pressure chamber with a glass window. The depth of fluid in the vial was about 2 mm at the lowest part of the meniscus. With this small amount of fluid, frequently shaken, rapid equilibration of liquid and gaseous phases was assured. Since bacterial luminescence is known to be highly insensitive to increased osmotic pressure, any slight evaporation was not important. Experimental temperatures were mostly between 23° and 25°C., occasionally 25.5°C; in any one experiment the variation was not more than 0.2° or 0.3°C.

Measurements of light intensity were made with the aid of a photomultiplier (R.C.A. 1 P 21), amplifier, and a milliammeter reading to 0.02 milliamperes. The photomultiplier tube was securely and tightly fitted in a block (at a constant distance from the material being measured) in which could be placed alternately the pressure chamber containing the experimental vial and the lucite adapter supporting the control vial. Between readings, the pressure chamber was placed in a mechanical shaker, and the control vial was shaken gently by hand.

Before starting an experiment the inlet tube to the pressure chamber was flushed out with the gas to be used, after which it was closed off. Each experiment consisted of three periods: (1) an initial period with air at atmospheric pressure in the pressure chamber; (2) an exposure period, during which the gas at the desired pressure was introduced as rapidly as possible; and (3) a recovery period in which the outlet cock and chamber were consecutively opened, the contents of the vial and chamber aerated, and the vial replaced in the chamber.

The curve relating time and light intensity of suspensions of luminescent bacteria in "PN solution" was found to be noticeably affected by the oxygen at high pressure, as illustrated by figure 1, in an experiment where 10 atmospheres of oxygen was used. In all cases the initial effect of the oxygen was found to be a swift, sharp drop in luminescence which progressed at a decreasing rate. After exposure there was a rapid increase of light production with return to the curve interrupted by the exposure period. After exposures of around 15 or 20 minutes, recovery was sometimes marked

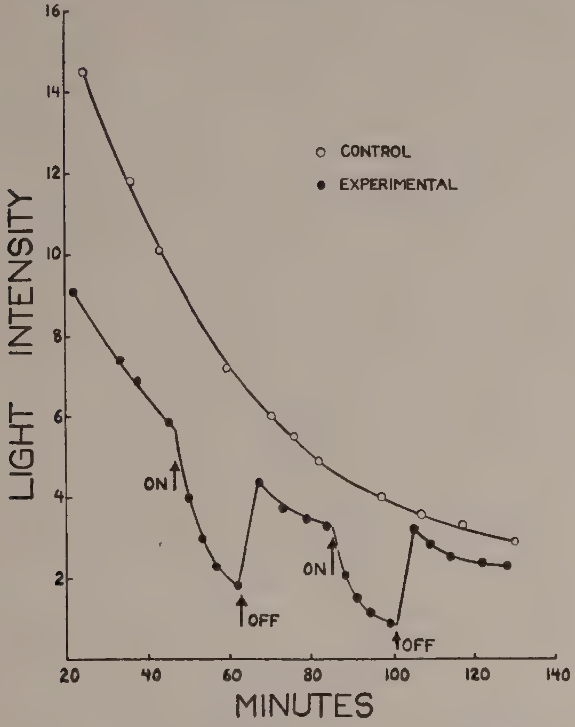

Fig. 1 The effect of 10 atmospheres of oxygen upon the light intensity of a suspension of Achromobacter fischeri.

Abscissa: Time in minutes

Ordinate: Light intensity in milliamperes

Note. The control suspension's curve is apparently higher because readings were not made through the pressure chamber window, as were those of the experimental suspension.

by a very slight, brief "overshooting" before the usual curve was resumed.

Figure 2 illustrates the relationship between oxygen pressure and its inhibitory effect in a series of experiments. In this graph the per cent to which light intensity was reduced after 10 minutes of exposure to oxygen is plotted against oxygen pressure. Although variations occur, the general trend is obvious. In spite of the relatively low threshold for oxygen toxicity, complete suppression of light intensity was not obtained, even at a pressure of ten atmospheres.

With the exception of one of the earlier experiments of a preliminary nature, reversibility was always obtained.

These results with high oxygen are not due to pressure per se, since such effects were never produced in other experiments using nitrogen up to pressures of 28 atmospheres in the presence of sufficient oxygen to maintain maximum luminescence. This ineffectiveness of nitrogen at 28 atmospheres is of further interest in showing that still higher pressures would be necessary if the so-called nitrogen narcosis were to be investigated in luminescent bacteria.

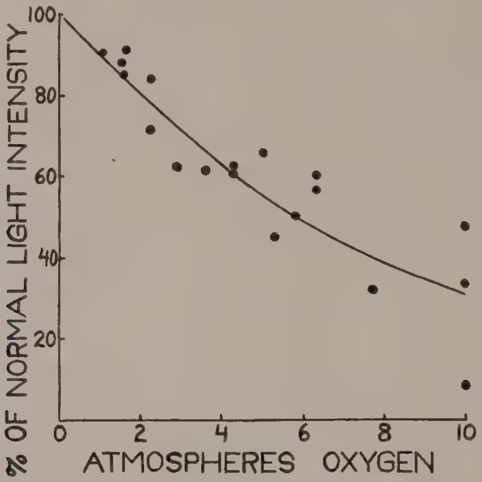

Fig. 2 The reduction of light intensity of suspensions of Achromobacter fischeri after 10 minutes of exposure to oxygen at various increased barometric pressures.

Abscissa: Oxygen pressure in atmospheres
Ordinate: Per cent of normal light intensity

SUMMARY

1. The light intensity of suspensions of Achromobacter fischeri was found to be reversibly diminished by increased oxygen pressures, up to the highest pressure tested, 10 atmospheres. Two atmospheres of oxygen or even less produced small but distinct effects, while greater pressures were correspondingly more inhibitory.

2. Reversal of this effect consisted of a resumption of the light intensity curve. After exposures of about 15 or 20 minutes it was sometimes marked by a very small, brief "overshooting."

3. Nitrogen gave no inhibitory effects at pressures up to 28 atmospheres.

We take pleasure in thanking Dr. J. J. Chang for valuable assistance in connection with the light measuring device.

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FLICKER IN ERYTHROCYTES. "VIBRATORY MOVEMENTS IN THE CYTOPLASM"?[1]

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There have been a number of recent accounts of a "vibratory movement in the cytoplasm of erythrocytes," (Pulvertaft, '49; Blowers et al., '51) and a brief historical note by Tompkins ('53). Under proper optical conditions (phase, darkfield and television microscopy) a normal erythrocyte appears to be undergoing a rhythmical and random alteration of optical density throughout its volume. This phenomenon is frequently called *flicker.*

The interpretation of this phenomenon offered by the foregoing authors is that the concentration of hemoglobin within the red cell varies from moment to moment in any given region of the cell and hence the observed optical density changes. Blowers et al. ('51), have presented data which they believe indicate that this phenomenon is linked to the metabolic (glycolytic) activity of the cell.

It is our purpose in this paper to present evidence which has forced us to the conclusion that this phenomenon is the resultant of Brownian motion, primarily of the whole cell and secondarily of its plasma membrane.

METHOD

Television microscopy, using either appochromatic or phase lenses, has been used (Parpart, '51; Parpart et al., '53). The red cells to be examined have for the most part been diluted

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in their own plasma and usually mounted on a special slide so that the dilute suspension of cells and plasma is completely covered by Shillaber oil and the oil immersion objective dipped into it. Occasionally, cells were mounted between slide and coverslip, the edges of the latter being paraffin sealed to prevent evaporation.

The "special slide" mentioned above consisted of a regular 1 × 3 inches microscope slide to the surface of which was sealed (by de Khotinsky cement) four lengths of 0.8 mm diameter glass capillary tubing in the form of a rectangle having dimensions of ca. $\frac{3}{4} \times 1\frac{1}{2}$ inches. The rectangular well thus formed served to contain the very thin film of plasma-diluted red cells spread over its bottom and the Shillaber oil that was quickly poured on top of it. By this means evaporation of water could be effectively checked. At the same time aqueous solutions of compounds whose effect on the *flicker* phenomenon it was desired to test could be readily introduced to the cells through this oil film. Another advantage of this slide is the fact that the sphering and/or crenation which commonly occurs to red cells suspended in saline solutions when mounted between slide and coverslip proceeds only very slowly on this type of slide.

The videcon tube used in the television camera had its maximum sensitivity in the range of 420 mμ and this sensitivity dropped to a low value at 500 mμ due to the properties of the tube and also dropped to a low value at 380 mμ due to the glass optics and slide employed. However, this resulted in a maximum of contrast for observation of the *flicker* effect, the Soret band of hemoglobin being at 413 mμ. The magnification, with 0.2 to 0.3 μ resolution, that we worked at on the viewing screen of the television monitor was 4,500 ×.

OBSERVATIONS, EXPERIMENTS AND DISCUSSION

A. The influence of Brownian motion on flicker

If the *flicker* effect is primarily associated with the random Brownian movement of the whole cell, then one might expect that decreasing the degree of freedom of motion of the cell would decrease the amount of observed *flicker*. That this is the case may be shown in the following types of experiments.

It must be borne in mind that in the following description the cells observed are normal biconcave discs.

Red cells (human) which are allowed to settle in their own plasma normally form rouleaux. The degree of *flicker* in such a mass of cells whether observed on edge or flat is always considerably less than is observed in the free, individual cell. Occasionally a red cell will attach to the end of a mass of rouleauxed cells with its long axis perpendicular to the long axis of the red cells composing the mass. Such a cell shows a small amount of *flicker* compared to the free cell. This is especially true when edge view comparison is made, since the teetering of the free cell on its edge just before it slowly tumbles on one of its greater surfaces, makes it appear as though it is behaving in a bellows-like manner. Under these conditions the *flicker* effect is at a maximum in the case of the free cell, whereas the red cell attached, as above described, to a rouleauxed mass of cells shows little of this "teeter" type of *flicker* activity.

The degree of freedom of Brownian motion of red cells can be decreased by increasing the viscosity or by causing a gelation of the surrounding medium. The effect of increased viscosity has been studied by adding a small amount of pure glycerol through the immersion oil *at one end* of the rectangular well of the special slide described under *Methods.* The glycerol was thus added to a cell preparation suspended in plasma which had previously been placed on the slide. In this manner a graded concentration of glycerol can be obtained ranging from pure glycerol at the end that it was introduced to no glycerol at the opposite end of the rectangular well. The degree of change of Brownian motion of the chylomicrons and platelets that are also present in the blood plasma can be used as an excellent criterion of the viscosity gradient. It has been observed that under these conditions in a region where the degree of motion of the platelets is greatly reduced and that of the chylomicrons slightly reduced, the degree of Brownian motion of the red cells is maximally reduced, and under these conditions the *flicker* phenomenon is absent.

The effect of gelation of the surrounding medium on *flicker* can be studied by suspending red cells in citrated plasma and then causing the plasma to clot by the addition of just sufficient calcium ions. As in the case of adding glycerol, the calcium is introduced as $CaCl_2$ solution through the Shillaber oil to a preparation of citrated plasma containing red cells. Prior to the addition the red cells show normal active *flicker*. Within a few minutes the long-fibrous clot can be seen forming and red cells trapped by these fibers always show a marked decrease in the amount of *flicker*. In a similar manner red cells suspended in a very thin sheet of a 5% gelatin gel, (made up in 1% NaCl-PO_4, pH 7.40), also show markedly decreased *flicker*.

Another method for mechanical immobilization of the red cell consisted of gradual compression between slide and coverslip by removal of plasma, and of course most of the cells, by means of filter paper wicks at the edge of the coverslip. The few remaining cells showed markedly decreased *flicker* as the cell's Brownian motion decreased as it became trapped but with no evidence of compression. Even the slightest compression stopped the *flicker* completely.

Thus far we have considered mechanical restriction of the whole cell from the point of view of changes in the environment. It is, however, possible to restrict the motion of the hemoglobin molecules within the cell. This can be accomplished by converting the cell's hemoglobin to the paracrystalline state. Rat red cells are particularly useful in this approach since their hemoglobin will crystallize within the cell when the environment is kept for about one hour at twice the normal tonicity. These cells retain their biconcave disc shape, though a few crenate, and yet the osmotic resistance is greatly increased, due to the lack of outward diffusibility of the semi-crystalline hemoglobin. Rat red cells treated in this way show only a slight decrease in the magnitude of the *flicker* phenomenon.

Under the conditions of the experiment just described we feel that we have thus demonstrated a separation of the two factors involved in the *flicker* phenomenon. In this experiment the Brownian motion of the whole cell would be only slightly altered and the *flicker* that was observed must have been due to this whole cell motion. On the other hand, the semi-rigid mass of hemoglobin within the cell would impede the motion of the plasma membrane that normally occurs and which would be reduced in this experiment and hence the slight decrease in *flicker* that was observed. The latter point of view depends on the fact that observations of the normal red cell by means of a phase microscope combined with a television pickup has shown that the plasma membrane is continuously being pushed in and out by molecular bombardment. Normally, then, the red cell membrane of some 60 A thickness (Parpart and Ballentine, '52), resembles a punching bag that is being repeatedly hit by many fists both from the inside and outside. This observed effect is most likely, though to a minor extent, a part of the cause of *flicker.*

Crystalline rat hemoglobin can be readily prepared by dropping packed rat red cells into a large volume (1–1000) of distilled H_2O. These crystals when of the size of a red cell show a similar type of *flicker,* associated with their thicker edges.

The result that *flicker* is only slightly decreased by causing the cell's hemoglobin to go into a semicrystalline state appears to be contrary to that speculated about by Tompkins ('53). However, the red cells she worked with became spheres under the experimental conditions. We have never observed *flicker* in sphered red cells, as will be detailed below.

B. *The influence of cell shape on flicker*

The evidence thus far presented strongly indicates that the primary causative agent for the *flicker* that can be observed in red cells is the Brownian motion of the whole cell. However, the biconcave disc shape of the red cell of most mammals is also an important factor. This has been ascertained by studying the influence of the shape of the cell on the degree of *flicker.*

Red cells of mammals may be turned into spheres either by adding a hemolytic agent (e.g. Rose Bengal, lecithin) to their environment or by decreasing the osmotic pressure of the environment, short of hemolysis. Such spherical red cells, where fine or coarse crenation is absent, never show the *flicker* phenomenon. Osmotically swollen cells, when reversed to the biconcave disc shape by bringing them into an isotonic environment, regain their normal type and degree of *flicker.* The work of Tompkins ('53) who sphered the red cell between slide and coverslip and found a loss of *flicker* in such cells parallels these observations. Our interpretation differs, however, since it is difficult to imagine that the hemoglobin in the cell that has become spherical in a non-hemolytic hypotonic salt solution, is in a paracrystalline state. Certainly polarization optical studies on such cells are not consistent with such an interpretation.

An added bit of evidence is the fact that if the red cell is hemolyzed under conditions where one-quarter of the hemoglobin is left in the cell and the cell brought back osmotically to its biconcave disc shape, this cell exhibits *flicker,* though less than with the normal amount of hemoglobin present.

Other shape changes from the normal biconcave disc shape affect *flicker.* Red cells immersed, as described under *Method,* in Hayem's solution become pancake shaped. This greatly reduces *flicker,* which is only observable near the rounded edge. This is what would be expected if Brownian motion of the whole cell plus the lens effect of the biconcave disc shape were the major causal agents of *flicker.* Homologous with this observation is the fact that nucleated red cells of other vertebrates show only a slight amount of *flicker* and this is confined to the edges of the cell. This is readily understandable in the above terms if it is recalled that these cells have a biconvex pancake shape. The normally pancake shaped ''red'' cell of the marine worm, Phascolosoma gouldi, also shows only slight, edge *flicker*.

C. Shape changes, metabolic inhibitors and flicker

Formalin dissolved in isotonic saline completely inhibits the metabolism of red cells. In concentration from 0.5 to 1% formalin, dissolved in isotonic saline, there is little or no shape change of the red cell and all of the cells show active *flicker*. A concentration of 5% formalin causes the red cells to assume a balloon tire-shape and show a very striking type of *flicker* which is confined to the inner and outer edges. Under these latter conditions if the balloon tire-shape cell is made to roll over so as to present edge view — the central *flicker* is very marked as the cell teeters back and forth.

Other inhibitors of glucose metabolism, e.g. sodium iodoacetate, sodium fluoride, do not abolish *flicker* even though the cell be exposed for a number of hours. At high concentration (0.01 M and 0.05 M respectively) crenation occurs and this alters the *flicker* but does not abolish it.

If whole rabbit blood is kept for six hours at 37°C. all of the glucose in the system is used up (Parpart and Green, '53). Such cells, diluted in the plasma in which they were allowed to stand for six hours at 37°C., show a normal degree and type of *flicker*. Addition of glucose (100 to 300 mg%), does not alter this normal *flicker*.

In fact a sample of centrifuged human red cells that had been autoclaved for two hours at 15 lbs. pressure was cooled and some of the blood sample mounted for examination. Surprisingly quite a few of these cells remained and many as good biconcave discs. These latter cells showed good, almost normal *flicker*.

Observations of the type just recorded make it impossible for us to believe the contention of Blowers et al. ('51) that ''*flicker* closely correlates with active cation transport.''

Crenated red cells, no matter by what method they may be formed, always show *flicker.* This is true whether the crenations are large blunt structures or fine crenation points. In fact the *flicker* at the base of a large blunt crenation point is more marked both with respect to increased optical density and rate of shifting of optical density, than in the normal biconcave disc. It is under these circumstances that the contribution of the very active Brownian motion of the red cell to the *flicker* phenomenon becomes most obvious. Another type of exaggeration of the *flicker* may be seen in freely suspended normal red cells when a platelet passes beneath the red cell. During this passage the amount of *flicker,* which in this case is also a purely optical effect, greatly increases in the regions of the red cell where the platelet, which is itself in active Brownian motion, casts its shadow at any particular moment.

RESUMÉ

The biconcave disc-shaped red cell of most mammals, when viewed by phase or television microscopy, can be observed to be in active Brownian motion. The natural and variable tilting of the whole cell that results from this motion, combined with the lens effect of the biconcave shape serves to reflect and refract light in a shimmering pattern over the entire cell. This, we are convinced from the evidence presented here, is the major cause of the phenomenon that has been termed *flicker.* The *flicker* effect becomes most prominent when the cell is rolled over so as to present an edge view to the observer. Under these conditions the teetering of the cell, under the influence of molecular bombardment, as it is slowly tumbling back to one of its greater surfaces, combined with the even more pronounced lens effect in edge view of a biconcave disc, gives the viewer the impression that the cell is acting in a bellows-like manner. This is associated with the broad shadows and shafts of light cast through the cell.

A secondary causal factor in the phenomenon of *flicker* are the minute outpushings and inpocketings that are visible all over the surface of the cell. These are continually fluctuating all over the surface and are most likely associated with the internal and external molecular bombardment of the 60 A thick plasma membrane of the red cell.

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